CANINE TOXICITY GENES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional application Serial No. 60/220,057, filed July 21, 2000 which is incorporated by reference in its entirety.

TECHNICAL FIELD

[0002] This invention is in the field of toxicology. More specifically, the invention provides for methods for identifying canine genes which are toxic response genes, the genes themselves, and methods of using these genes. Also provided herein are primer sequences and sequence of canine genes which are useful for making and using arrays to determining toxicological responses to various agents and also useful for identifying novel canine gene sequences and novel canine genes.

BACKGROUND OF THE INVENTION

[0003] Individuals exhibit a high degree of variability in response to agents such as drugs, pharmaceutical compounds, and chemicals. The development of a drug or pharmaceutical compounds can take many years and cost millions of dollars. In addition, some companies use animals (*e.g.*, mice, rabbits, dogs, cats, pigs, etc.) to test the efficacy and toxicity of drugs and/or pharmaceutical compounds to obtain data for Phase I trials. Many drugs that are being developed do not proceed beyond a Phase I trial for many different reasons. One plausible reason is a lack of data in an accepted animal model for the disease or symptoms which the drug is targeted to treat. Animals used in various disease models include, but are not limited to dogs, pigs, rabbits, cats, chimpanzees, and other primates. In addition, animals are used to test toxicity levels and toxicological responses to drugs and pharmaceutical compounds under development.

[0004] In animals, toxicity of a drug can be determined by observing several *in vivo* parameters, including but not limited to drug levels in blood, tissues, urine, and other biological fluids; enzymatic levels in tissues and organs; protein or sugar

levels in blood and other biological fluids; elevation or depression in number, size, morphology, and/or function of cells (*e.g.*, white blood cells, lymphocytes, red blood cells, etc.), tissues, or organs (*e.g.*, liver, heart, kidney, etc.). Other physical and physiological parameters which may be useful include but are not limited to survival rate of animals, appearance (*e.g.*, hair loss, brightness of eyes, etc.), and behavior (*e.g.*, eating habits, sleeping habits, etc.).

[0005] With the advent of molecular and recombinant technology, genetic and molecular analysis provides another method by which toxicity may be measured. Differential gene expression technology involves detecting the change in gene expression of cells exposed to various stimuli. The stimulus can be in the form of growth factors, receptor-ligand binding, transcription factors, or exogenous factors such as drugs, chemicals, or pharmaceutical compounds. Differential gene expression can be observed by using techniques involving gel electrophoresis and polynucleotide microarrays.

[0006] A polynucleotide microarray may include genes for which full-length cDNAs have been accurately sequenced and genes which may be defined by high-throughput, single-pass sequencing of random cDNA clones to generate expressed sequence tags (ESTs). Bioinformatic algorithms such as Unigene group cDNA clones with common 3' ends into clusters which tentatively define distinct human genes. An ideal cDNA microarray might therefore contain one representative from each Unigene cluster. In practice, given the current complement of about 45,925 Unigene clusters, most microarrays contain at most one-third of the total Unigene set.

[0007] Researchers focused on detecting changes in expression of individual mRNAs can use different methods to detect changes in gene expression, for example, microarray, gel electrophoresis, etc. Other methods have focused on using the polymerase chain reaction (PCR) and/or reverse transcriptase polymerase chain reaction (RT-PCR) to define tags and to attempt to detect differentially expressed genes. Many groups have used PCR methods to establish databases of mRNA

sequence tags which could conceivably be used to compare gene expression among different tissues (See, for example, Williams, J. G. K., *Nucl. Acids Res.* 18:6531, 1990; Welsh, J., et al. *Nucl. Acids Res.*, 18:7213, 1990; Woodward, S. R., Mamm. *Genome*, 3:73, 1992; and Nadeau, J. H., Màmm. *Genome* 3:55, 1992). This method has also been adapted to compare mRNA populations in a process called mRNA differential display. In this method, the results of PCR synthesis are subjected to gel electrophoresis, and the bands produced by two or more mRNA populations are compared. Bands present on an autoradiograph of one gel from one mRNA population, and not present on another, correspond to the presence of a particular mRNA in one population and not in the other, and thus indicate a gene that is likely to be differentially expressed. Messenger RNA derived from two different types of cells can be compared by using arbitrary oligonucleotide sequences of ten nucleotides (random 10-mers) as a 5' primer and a set of 12 oligonucleotides complimentary to the poly A tail as a 3' "anchor primer". These primers are then used to amplify partial sequences of mRNAs with the addition of radioactive deoxyribonucleotides. These amplified sequences are then resolved on a sequencing gel such that each sequencing gel has a sequence of 50-100 mRNAs. The sequencing gels are then compared to each other to determine which amplified segments are expressed differentially (See, for example, Liang, P. et al. Science 257:967, 1992; See also Welsh, J. et al., Nucl. Acid Res. 20:4965, 1992; Liang, P., et al., Nucl. Acids Res., 3269 1993; and U.S. Patent Nos. 6,114,114 and 6,228,589).

[0008] The process of isolating mRNA from cells or tissues exposed to a stimulus (*e.g.*, drugs or chemicals) and analyzing the expression with gel electrophoresis can be laborious and tedious. To that end, microarray technology provides a faster and more efficient method of detecting differential gene expression. Differential gene expression analysis by microarrays involves nucleotides immobilized on a substrate whereby nucleotides from cells which have been exposed to a stimulus can be contacted with the immobilized nucleotides to generate a

hybridization pattern. This microarray technology has been used for detecting secretion and membrane-associated gene products, collecting pharmacological information about cancer, stage specific gene expression in Plasmodium falciparum malaria, translation products in eukaryotes, and a number of other scientific inquiries. See, for example, Diehn M, et al. *Nat Genet.* 25(1): 58-62 (1993); Scherf, U., et al. *Nat Genet.* 24(3): 236-44 (1993); Hayward R.E., et al. *Mol Microbiol* 35(1): 6-14 (1993); Johannes G., et al. *Proc Natl Acad Sci U S A* 96(23): 13118-23 (1993). Microarray technology has also been used in exploring drug-induced alterations in gene expression in Mycobacterium tuberculosis. See, for example, Wilson M., et al. *Proc Natl Acad Sci.* 96(22): 12833-8 (1999). The use of microarray technology with animal genes, *e.g.,* canine genes, during drug development to detect drug-induced alternation in vertebrates, such as dogs, would provide a method that is fast, efficient, cost-effective and could spare many animals from being the subjects of laboratory tests.

[0009] The discovery and/or characterization of a set of toxicologically relevant genes would be useful in simplifying the development, screening, and testing of new drugs. While some genes are known to be differentially displayed in response to one agent, a more useful tool for assessing toxicity is a panel of genes which are identified as toxicologically relevant genes. The invention provided herein fulfills these needs and provides disclosure to novel canine genes as well.

[0010] The disclosure of all patents and publications cited herein are hereby incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

[0011] Disclosed herein are methods of identifying and isolating canine genes which are toxicologically relevant and methods of using these toxicologically relevant canine genes to determine toxic responses to an agent. Further, arrays containing the canine genes, methods of making these arrays, and methods of using these arrays are provided. Also disclosed herein are primer sequences useful for

obtaining canine genes, which in turn have a variety of uses, and gene sequences for novel canine genes discovered using these methods.

[0012] In one aspect, a method of identifying a toxicologically relevant canine gene is disclosed whereby the gene expression profile of untreated canine cells is obtained as well as a gene expression profile of canine cells treated with an agent. The gene expression profile of untreated canine cell is compared with the gene expression profile of the treated canine cells to obtain a gene expression profile indicative of a toxicological response. In some aspects, canine cells can be any type of cells including but not limited to biological samples from liver, lung, heart, kidney, spleen, testes, thymus, brain, or cells lines obtained from commercial sources (*e.g.*, ATCC). The agent can be any type of synthetic or non-synthetic compound including but not limited to agents listed in Table 10.

[0013] In another aspect, a method of isolating canine genes indicative of a toxicological response to an agent is provided wherein sequences of mammalian, non-canine genes associated with toxicological responses are provided, primers homologous to said genes associated with toxicological responses are provided; and the primers are used to amplify canine genes from canine cDNA library.

[0014] In yet another aspect, a method for determining a toxicological response to an agent is provided wherein cells are exposed to an agent and a first gene expression profile is obtained and then compared to a gene expression profile of toxicologically relevant canine genes to determine if the first gene expression profile is indicative of a toxicological response. In one aspect, the gene expression profiles of one or more toxicologically relevant canine gene(s) are stored in a database. In another aspect, a database containing multiple gene expression profiles of toxicologically relevant canine genes is used.

[0015] In yet another aspect, a method for determining a toxicological response to an agent in an organ is provided wherein cells are exposed to an agent and a gene expression profile is obtained and then compared to a gene expression

profile of toxicologically relevant canine genes to determine if the first gene expression profile is indicative of a toxicological response in an organ.

[0016] In another aspect, a method for screening an agent (*e.g.*, drug, medicament, or pharmaceutical composition) for potential toxicological responses is provided wherein cells are exposed to an agent; and a gene expression profile is obtained and then compared to a gene expression profile of toxicologically relevant canine genes to determine if the first gene expression profile is indicative of a toxicological response in genes associated with toxicological responses. In one aspect, a database containing at least one gene expression profile of toxicologically relevant canine genes is used for comparison.

[0017] In one aspect, the invention relates to methods of identifying canine genes and gene sequences which are indicative of a toxicological response. These genes and their gene expression profiles are stored in a database. The database is useful for toxicological studies and analysis, particular when applied to the screening, development, and testing of potential new drugs. A panel of genes indicative of toxicity can vary between organs different in time of exposure to one or more agents resulting effects of agent(s) and, different compounds. In one aspect, the canine genes and gene sequences identified to be indicative of toxicological response (*i.e.*, toxicologically relevant) are novel.

[0018] In another aspect, a method for generating a canine array comprising at least ten canine genes which are indicative of a toxicological response is provided. Genes indicative of toxicological response are immobilized to a substrate.

[0019] In another aspect, an array is provided comprising at least ten canine toxicological response genes or a portion thereof immobilized on a substrate. The canine genes are assembled in an array such that at least 2 genes, more preferably at least 5 genes, more preferably at least 10 genes, more preferably at least 20 genes, more preferably at least 30 genes, even more preferably at least 40 genes, more preferably at least 50 genes, more preferably at least 100 genes, more preferably at

least 250 genes, more preferably at least 400 genes, more preferably at least 500 genes, more preferably at least 600 genes, more preferably at least 750 genes, more preferably at least 850 genes, and more preferably at least 1000 genes are assembled on such array. In one aspect, the toxicologically relevant genes are attached to the array substrate by covalent linkage. In another aspect, the genes or portions thereof are capable of hybridization to expressed nucleic acids derived from a cell and are capable of indicating a toxicological response of the cell to said agent.

[0020] In yet another aspect, a method for obtaining a gene expression profile is provided whereby a population of cells is exposed to an agent, cDNA from the population of cells is obtained, labeled, and contacted with the array comprising toxicologically relevant genes.

[0021] In still another aspect of the invention, primer sequences that are used for identifying canine genes are disclosed. These primer sequences can be used for probes, for PCR-related amplification, included on an array chip for identifying nucleotide sequences related to toxicological responses, or for identifying novel canine genes. Sequences of such primers and methods of using thereof are disclosed herein.

[0022] In yet another aspect of the invention, novel canine genes or portions of novel canine genes are disclosed and uses thereof. The sequences of novel canine genes are disclosed in Table 8. In one aspect, an array comprising at least 2, 5, 10, 25, 50, or 56 novel canine toxicologically relevant genes from Table 8 is provided. In one aspect, an array comprising at least 2, 5, 10, 25, 50, 60, 75, 90, 100, or 116 novel canine toxicologically relevant genes from Table 9 is provided.

[0023] In yet another aspect, novel canine sequences are cloned and/or maintained in expression vectors. In one aspect, novel canine sequences which are cloned in expression vectors are expressed and/or maintained in suitable eukaryotic host cells.

BRIEF DESCRIPTION OF THE TABLES

[0024] Table 1 depicts the primers used to isolate toxicologically relevant canine genes from a canine cDNA library.

[0025] Table 2 depicts target sequences obtained by using primers listed in Table 1.

[0026] Table 3 depicts 50-mer sequences for toxicologically relevant canine genes.

[0027] Table 4 depicts the accession numbers which correspond with toxicologically relevant canine genes.

[0028] Table 5 depicts toxicologically relevant genes that were identified and isolated using differential display.

[0029] Table 6 depicts canine genes that are identified and isolated by using primers to known toxicologically relevant human genes.

[0030] Table 7 depicts canine genes which have been identified as toxicologically relevant by differential display.

[0031] Table 8 depicts canine genes which have not been disclosed in a public sequence database, printed publications, or scientific conferences.

[0032] Table 9 depicts canine genes which are associated with specific agents.

[0033] Table 10 provides a list of agents which are used or can be used to determine toxicologically relevant canine genes.

[0034] Table 11 depicts the fold induction for canine genes in a canine array.

BRIEF DESCRIPTION OF THE FIGURES

[0035] The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of necessary fee.

[0036] Figure 1 is a scan of a microarray which shows toxicological responses to three doses of cadmium chloride using canine arrays.

[0037] Figure 2 a chart which shows the fold induction in an analysis of toxicological responses to cadmium chloride using canine arrays.

[0038] Figure 3 is a graph which shows dose response of interleukin-8.

[0039] Figure 4 is a scan of a microarray which shows the gene expression profile of a canine liver dosed with erythromycin estolate (100mg/kg).

[0040] Figure 5 is a scan of a microarray which shows the gene expression profile of a canine kidney dosed with erythromycin estolate (100mg/kg).

[0041] Figure 6 is a diagram of first strand synthesis for the design of an antisense probe from amplified antisense RNA for hybridization to microarrays with sense targets.

[0042] Figure 7 is a diagram of second strand synthesis for the design of an antisense probe from amplified antisense RNA for hybridization to microarrays with sense targets.

[0043] Figure 8 is a diagram of antisense probe synthesis for the design of an antisense probe from amplified antisense RNA for hybridization to microarrays with sense targets.

DETAILED DESCRIPTION OF THE INVENTION

[0044] The present invention discloses canine genes which are indicative of a toxicological response. Methods of identifying genes indicative of a toxicological response and isolating such genes are provided. Novel canine genes and methods of isolating the novel canine genes, including primers used, and methods of identifying them are also provided. Further, arrays which comprise canine genes for use in detecting gene expression indicative of toxicological response, the methods of making the array, and the canine genes which are included in the array are also provided.

General Techniques

[0045] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant

techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook *et al.*, 1989); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Animal Cell Culture* (R.I. Freshney, ed., 1987); *Handbook of Experimental Immunology* (D.M. Weir & C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller & M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M. Ausubel *et al.*, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J.E. Coligan *et al.*, eds., 1991); *The Immunoassay Handbook* (David Wild, ed., Stockton Press NY, 1994); *Antibodies: A Laboratory Manual* (Harlow *et al.*, eds., 1987); *Methods of Immunological Analysis* (R. Masseyeff, W.H. Albert, and N.A. Staines, eds., Weinheim: VCH Verlags gesellschaft mbH, 1993); *Principals and Methods in Toxicology* (A. Wallace Hayes, ed., 2000); *Analytical Methods in Toxicology* (H.M. Stahr, 1991); and *PCR Protocols in Molecular Toxicology* (John P. Vanden Heuvel, ed., 1997).

Definitions

[0046] "Toxicity", as used herein, refers to the exaggerated micro- or macroscopic responses of cells, tissues, organs or systems to low, average, or high doses of an agent. These responses may lead to observable symptoms such as dizziness or nausea and can also result in toxic outcomes. Toxicity often results in toxic side effects that are different, in either degree or kind, from the response of the majority of patients at the recommended dose. Toxicity may be characterized by, but is not limited to, the differential expression of genes when compared to the response of a similar individual who is not exposed to a given agent.

[0047] A "toxicological response", or "toxic response" used interchangeably herein, refers to a cellular, tissue, organ or system level response to exposure to an agent and includes, but is not limited to, the differential expression of genes and/or proteins encompassing both the up- and down-regulation of such genes; the up- or

down-regulation of genes which encode proteins associated with the repair or regulation of cell damage; or the regulation of genes which respond to the presence of an agent.

[0048] The terms "toxicologically relevant gene", "toxicity gene", and "toxic response gene" are interchangeable as used herein. A toxic response gene can be defined as a gene whose message or protein level is altered by adverse stimuli (e.g, an agent). The specific set of genes that cells induce is dependent upon the type of damage or toxic threat caused by the agent and which organs are most threatened. In addition to the up-regulation of genes which respond to specific toxic threat, genes which encode functions not appropriate under conditions of toxic injury may be down regulated.

[0049] As used herein, "toxic outcome" refers to the cellular, molecular microscopic or macroscopic, molecular symptoms, physiological, morphological or pathological changes which are observed as a result of exposure to an agent.

[0050] As used herein, the term "agent" means a biological or chemical compound such as a simple or complex organic or inorganic molecule, a peptide, a protein, an oligonucleotide, an antibody, an antibody derivative, or antibody fragment. Various compounds can be synthesized, for example oligomers, such as oligopeptides and oligonucleotides, and synthetic organic compounds based on various core structures, and these are also included in the term "agent". In addition, various natural sources can provide compounds for screening, such as plant or animal extracts, and the like. Agents can be tested and/or used singly or in combination with one another. An "agent" to which an individual has a toxicological response can also be any substance to which an individual exhibits a toxicological response and includes, but is not limited to, drugs, pharmaceutical compounds, household chemicals, industrial chemicals, environmental chemicals, and other chemicals and compounds to which individuals may be exposed. Exposure to an agent can

constitute physical contact as well as secondary contact, such as inhalation and environmental exposure.

[0051] As used herein, the term "gene" refers to polynucleotide sequences which encode protein products and encompass RNA, mRNA, cDNA, single stranded DNA, double stranded DNA and fragments thereof. Genes can include introns and exons. It is understood that the polynucleotide sequences of a gene can include complimentary sequences (*e.g.*, cDNA).

[0052] The term "gene sequence(s)" refers to gene(s), full-length genes or any portion thereof.

[0053] The term "novel gene" refers to a gene and/or gene sequences that have not been disclosed in public sequence databases, in any printed publication, or a public forum (*e.g.*, scientific conferences) as of June 2001.

[0054] "Differential expression" as used herein refers to the change in expression levels of genes, and/or proteins encoded by said genes, in cells, tissues, organs or systems upon exposure to an agent. As used herein, differential gene expression includes differential transcription and translation, as well as message stabilization. Differential gene expression encompasses both up- and down-regulation of gene expression.

[0055] "Gene expression indicative of toxicological response", as used herein, refers to the relative levels of expression of a gene, for example a toxic response gene. Profiles of gene expression profiles may be measured in a sample, such as samples comprising a variety of cell types and may, for example, comprise blood, urine, spinal fluid or serum.

[0056] The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified,

non-natural or derivatized nucleotide bases. It is understood that the double stranded polynucleotide sequences described herein also include the modifications described herein. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a oligodeoxynucleoside phosphoramidate (P-NH2) or a mixed phosphoramidate-phosphodiester oligomer. A phosphorothioate linkage can be used in place of a phosphodiester linkage. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand *de novo* using a DNA polymerase with an appropriate primer. The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

[0057] As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides.

[0058] "Naturally occurring" refers to an endogenous chemical moiety, such as a carbohydrate, polynucleotide or polypeptide sequence, *i.e.*, one found in nature. Processing of naturally occurring moieties can occur in one or more steps, and these terms encompass all stages of processing. Conversely, a "non-naturally occurring" moiety refers to all other moieties, *e.g.*, ones which do not occur in nature, such as recombinant polynucleotide sequences and non-naturally occurring carbohydrates.

[0059] A polynucleotide is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to produce the polypeptide or a fragment thereof. For purposes of this invention, and to avoid cumbersome referrals to complementary strands, the anti-sense (or complementary) strand of such a polynucleotide is also said to encode the sequence; that is, a polynucleotide sequence that "encodes" a polypeptide includes both the conventional coding strand and the complementary sequence (or strand).

[0060] "Hybridization" or "hybridize" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding is sequence-specific, and typically occurs by Watson-Crick base pairing. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR, or the enzymatic cleavage of a polynucleotide by a ribozyme.

[0061] Hybridization reactions can be performed under conditions of different "stringency". Relevant conditions include temperature, ionic strength, time of incubation, the presence of additional solutes in the reaction mixture such as formamide, and the washing procedure. Higher stringency conditions are those conditions, such as higher temperature and lower sodium ion concentration, which require higher minimum complementarity between hybridizing elements for a stable hybridization complex to form. Conditions that increase the stringency of a hybridization reaction are widely known and published in the art: see, for example, "Molecular Cloning: A Laboratory Manual", Second Edition (Sambrook, Fritsch & Maniatis, 1989). When hybridization occurs in an antiparallel configuration between two single-stranded polynucleotides, those polynucleotides are described as "complementary". A double-stranded polynucleotide can be "complementary" to another polynucleotide, if hybridization can occur between one of the strands of the first polynucleotide and the second. Complementarity (the degree that one

polynucleotide is complementary with another) is quantifiable in terms of the proportion of bases in opposing strands that are expected to form hydrogen bonding with each other, according to generally accepted base-pairing rules.

[0062] A "host cell" includes an individual cell or cell culture which can be or has been a recipient for vector(s) or for incorporation of polynucleotides and/or proteins. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in genomic of total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. A host cell includes cells transfected in vivo with a polynucleotide(s) of this invention.

[0063] "Transformation" or "transfection" refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, lipofection, transduction, infection or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host cell genome.

[0064] The terms "protein", "polypeptide", and "peptide" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. It also may be modified naturally or by intervention; for example, disulfide bond formation, glycosylation, myristylation, acetylation, alkylation, phosphorylation or dephosphorylation. Also included within the definition are polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids) as well as other modifications known in the art.

[0065] As used herein, "array" and "microarray" are interchangeable and refer to an arrangement of a collection of nucleic acids (*e.g.*, nucleotide sequences) in a centralized location. Arrays can be on a solid substrate, such as a glass slide, or on a semi-solid substrate, such as nitrocellulose membrane. The nucleotide sequences

can be DNA, RNA, or any combination or permutations thereof. The nucleotide sequences can also be partial sequences from a gene, primers, whole gene sequences, non-coding sequences, coding sequences, published sequences, known sequences, or novel sequences.

[0066] An "individual" is a vertebrate, preferably a mammal, for example a dog. Mammals include, but are not limited to, humans, farm animals, sport animals, pets, primates, mice, and rats.

[0067] A "biological sample" encompasses a variety of sample types obtained from an individual. Biological samples or "samples" can be used in various manners (*e.g.*, in the determination of toxicological response, analysis of one or more toxicological responses, etc.). The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen, whole organs, tissue cultures or cells derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with agents, reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

Methods of the Invention

[0068] Canine genes which are toxicologically relevant have been identified and disclosed herein. In some embodiments, canine genes which are disclosed are novel. Methods of identifying toxicologically relevant genes are described herein. In addition, methods of isolating and using toxicologically relevant genes are disclosed. In one embodiment, toxicologically relevant genes are used to make arrays. The arrays can be used for drug screening purposes to determine toxicological response.

Methods of identifying a set of toxicologically relevant genes

[0069] Identification of a set of toxicologically relevant genes can be achieved by several methods. One method which can be used is to clone genes

previously described to be relevant in toxicology. Using published sequences, for example in literature or from GenBank, primers can be made and then used to PCR amplify from a relevant cDNA library to obtain the toxicologically relevant gene of interest which can then be cloned into a plasmid or an expression vector, depending on the use desired. The gene, *i.e.*, nucleic acid, can be placed amongst other toxicologically relevant genes in an microarray for high-throughput testing, as disclosed *infra*. For example, using known toxicologically relevant canine sequences, primers can be designed and used in PCR reaction to amplify the canine gene from a cDNA library. The cDNA library can be made from different canine cells. In one embodiment, primary liver cells from a beagle are used as source for the cDNA library. The generation of a cDNA involves reverse transcribing isolated RNA and is well known in the art (see for example, Sambrook et al. *supra*). The canine gene fragments, amplified by PCR, are cloned into any standard plasmid expression vector which can be obtained from numerous commercial sources (*e.g.*, Promega, InVitrogen, New England BioLabs, etc.) and sequenced. The resulting sequence information is then compared to the GenBank database to confirm that the cloned DNA is the specific canine gene for which the primers were designed. Upon positive confirmation of the sequence, the amplified gene is then added to the panel of genes to be included in the array. Methods of including toxicologically relevant genes in an array are disclosed below.

[0070] Alternatively, for replication to high copy numbers, a plasmid may be used to grow high copies of the toxicologically relevant gene of interest which can then be purified by any commercially available kit (*e.g.*, from Qiagen or Promega). The purified toxicologically relevant gene may be used for "spotting" in a microarray or alternatively, the purified nucleic acid can then be inserted into an expression vector, transfected into mammalian cells, *e.g.*, canine cells, and then the cells can be exposed to a compound and observed for toxicological responses. Toxicity may be ascertained by observing changes in cell morphology or re-arrangement of

cytoskeleton, which can be determined by examination under a microscope, or alternatively, cell apoptosis or necrosis. In another alternative, "transcriptome profiling", described in greater detail below, may be used whereby nucleic acid can be isolated from both the exposed and unexposed cells and examined to determine which level of the compound causes the up-regulation or down-regulation of the toxicologically relevant gene of interest.

[0071] Another method which may be used to identify canine genes utilizes known sequences of toxicologically relevant non-canine (*e.g.*, human) genes. These toxicologically relevant genes may be from a non-canine species including, but not limited to humans, primates, and other mammals. Primers to these toxicologically relevant non-canine genes are designed, synthesized, and are subsequently used in PCR reaction with canine cDNA libraries to amplify the homologous canine gene. The homologous canine gene may or may not be the exact sequence as the non-canine gene with which the primers were designed. It is understood that some changes in the nucleotide sequence can occur and the homologous canine gene can still be toxicologically relevant and/or retain the same function as the non-canine gene. The amplified canine genes can then be added to the panel of genes to be included in the array.

[0072] In yet another embodiment, target sequences for inclusion in a canine array are obtained by *de novo* synthesis of nucleotides and then immobilization on a substrate, *e.g.*, a glass slide. The target sequences are from genes which can indicate one or more toxicological responses. Target sequences exemplifying this embodiment are shown in Table 3.

[0073] Another method which can be used to identify a set of toxicologically relevant genes is to analyze the gene expression profile from tissues in canine toxicity studies and select those genes with differential expression. Differential expression may be assessed by any number of methods. One method which may be used is by microarray analysis. Provided herein are methods of using microarray analysis to

determine differential gene expression. Another method of determining differential gene expression is by reverse transcriptase-polymerase chain reaction (RT-PCR), *e.g.*, Taqman® technology (Foster City, CA). Yet another method which could be used to detect differential gene expression is Invader® technology, commercially available from Third Wave (Madison, WI). Yet another method which may be used to determine differential expression is Northern blot analysis.

[0074] Other methods which may be used include open systems such as AFLP and SAGE (Klein, P.E., et al. *Genome Res.* 10(6):789-807 (2000); Wang, X. and Feuerstein, G.Z., *Cardiovasc Res.* 35(3):414-21 (1997)) Feuerstein, G.Z. and Wang X. *Can J. Physiol Pharmacol.* 75(6):731-4 (1997); Hough, C.D. et al., *Cancer Res.* 60(22):6281-7 (2000); Ye, S.Q., et al., *Anal Biochem.* 287(1):144-52 (2000)). An "open system" allows the entire transcriptome to be analyzed instead of a defined set of genes.

[0075] Alternatively, comparisons between gene expression profiles from control canine cells (or canine cell lines) and canine cells (or canine cell lines) treated with an agent can be used to select responsive genes. This is referred to herein as "transcriptome profiling". This method empirically determines which genes are toxicologically relevant by analyzing differential gene expression. In this embodiment, experimental canines are divided into two groups. One group is exposed to one agent (*e.g.*, with a suitable vehicle) at different concentrations for different lengths of time. Another group of canines is exposed to vehicle only and serves as the control group. Canines are then sacrificed and organs such as liver, spleen, kidney, testes, heart, and thymus are harvested for cells to perform molecular analysis of gene expression. In addition, analysis of serum proteins in the circulating blood can provide another measure to compare with unexposed canines. Once the experimental group is exposed to at least one agent, then RNA of both groups is isolated and reverse transcribed in PCR reactions to generate cDNA which in turn is amplified to generate double stranded DNA. The PCR is performed in the presence

of a radioactive or fluorescent DNA substrate that is incorporated into the double stranded DNA. On a polyacrylamide gel, the DNA derived from the treated cells is separated by length next to the DNA derived from untreated population. The intensity of the resulting band or bands is compared between the treated and untreated groups of cells. Bands that show different radioactive or fluorescent intensity are excised from the gel, amplified by PCR, cloned, and sequenced, as disclosed herein. The sequences are compared with known gene sequences in the public databases such as GenBank. In this manner, novel canine genes, in addition to known canine genes with varying degrees of similarity, which are toxicologically relevant are discovered and identified. The examples disclosed herein illustrates how this aspect of the invention may be practiced by the skilled artisan.

[0076] If a partial sequence of a novel canine gene is discovered, the technology, texts (see Sambrook et al. *infra*), and resources available to a skilled artisan would enable the skilled artisan to sequence the remainder of the gene and obtain a full-length gene without undue experimentation. One method of obtaining the remaining portion of a novel canine gene is to make primers corresponding to the part of the novel canine gene which are known combined with random primers and then use the primers in PCR reactions with a canine cDNA library. The PCR reaction are run on a standard agarose gel and amplified bands are identified, excised from the gel, and sequenced.

[0077] Other factors to consider in identifying toxicologically relevant genes include, but are not limited to, selection of one or more agent(s), the dosage amount to administer, routes of administration, time of exposure, and metabolism of the agent.

Selection of agent(s)

[0078] The agent to be tested can selected on the basis of different criteria. One method of selecting which compound to test is damage observed in specific organs. For example, cisplatin, amphotericin B and gentamicin have been observed

to cause kidney tubular epithelial cell damage. Another example, liver peroxisome proliferation has been observed to be affected by clofibrate, gemfibrozil, and WY 14643. Another basis for selection is function. For example, cisplatin causes apoptosis and reactive oxygen species, amphotericin B causes increased permeability of cell membranes to ions and renal vasoconstriction, and gentamicin causes phospholipid accumulation in lysosomes.

[0079] Other toxicants affect an organ in general, for example, some kidney toxicants include but are not limited to cisplatin, gentamicin, puromycin, and amphotericin B. Liver toxicant include but are not limited to chlorpromazine, clofibrate, diflunisal, tetracycline, erythromycin, and ethanol. Immunotoxicants include but are not limited to cyclosporin A, lipopolysaccharide (LPS), hydroxyurea, phenylhydrazine, dexamethasone, estradiol, and tamoxifen. Heart toxicant includes but is not limited to doxorubicin. Multiorgan toxicants include but are not limited to methotrexate and cadmium chloride.

[0080] Other criteria for selecting an agent to test is to select those agents to which an individual might be exposed to on a regular basis, either in the environment, by prescription or over-the-counter drug. Another criteria for selecting an agent is the need to obtain toxicity information for FDA-approval or alternatively for other toxicity requirements, for example in pre-clinical or clinical trials.

Determination of dosage

[0081] Dosages to use in canine experiments can be determined using several methods. One method is to use reported dosages as a starting point and dose incrementally above and below the reported dosage. Increments can be at least ±1%, 5%, 10%, 25%, 35%, 45%, 50%, 60%, 70%, 80%, 90%, or 95%. Upregulation or downregulation of markers in the blood including but not limited to serum chemistry values and hematology values can be used to determine if toxicity has been reached. Alternatively, examining the histopathology of organs, in particular, organs which are the specific targets of the compound of interest, may be used to determine if a

pathological change has occurred in response to administration of the compound. Another method which may be used is to determine the molecular changes by analyzing the gene expression in response to administration of different doses of a compound by the methods disclosed *infra*.

[0082] Determination of the dosage experimentally using cell cultures is affected by many factors: the nature of the agent, its potency, mechanism of action, type of cell which is the target of the agent, and number of cells. To determine the dosage required experimentally, a low dosage level of the agent is added and then in a step-wise manner, the dosage is increased as well as length of time exposed to the agent. If the agent is lipophilic and easily crosses the lipid bilayer of cells, a lower initial concentration may be used and/or shorter length of time exposed to the agent. If the agent has the property of not being able cross the cell barrier easily (*e.g.*, lipophobic) and would need to be actively or passively transported across cell membranes, then a slighter higher initial concentration may be used and/or longer length of time exposed to the agent. Increasing dosage step-wise while monitoring toxicological response and morphology of the cells, rate of death of the cells, and growth patterns allows the skilled artisan to determine the dosage at which a toxicological response occurs. However, it should be noted that toxicological responses may occur which are visible changes, including but not limited to, physical structure and integrity of the cells (i.e. morphology, growth pattern, etc.). Monitoring for cellular toxic responses as well as molecular toxic responses, *e.g.*, differential gene expression increases the likelihood of finding preferable dosages. Combining visualization as well as monitoring for cellular changes and molecular changes (*e.g.*, differential gene expression) increases the likelihood of finding preferable dosages.

[0083] Changes in gene expression may be toxicologically significant. The point at which toxicologically relevant gene expression becomes even more relevant is at that dosage at which removal or diminishment of the treatment no longer results in a return to normalcy, *i.e.*, the state of a cell, organ, or system that existed prior to

the treatment with the compound. Treatments beyond a certain dosages or time period may commit the cell to a toxicologically relevant fate. This toxic dosage will be reflected by an identifiable gene expression pattern, which will be distinct from the pattern observed below the toxic dosage.

[0084] Dosage response is an important concept in toxicology. Depending on the dosage of a toxin or agent which may be toxic, the gene expression profile of a particular gene may vary. One way that this can be envisioned is by observing the changes in fold induction of a particular gene when analyzed using the arrays of this invention. The dosages determined in dose response curves may be useful in determining "threshold" levels of toxicity, for example for FDA approval. Example 15 and Figure 3 illustrate this embodiment. Methods of analyzing gene expression and how to correlate gene expression data are provided herein.

Administration of an agent

[0085] Administration of one or more agents to dogs may be achieved by various routes. It will be readily appreciated by those skilled in the art that the route can vary, and can be intraperitoneal, intravenous, subcutaneous, topical, transcutaneously, intramuscular, enterally, transdermally, transmucously, sustained release polymer compositions (*e.g.*, a lactide polymer or co-polymer microparticle or implant), perfusion, pulmonary (*e.g.*, inhalation), nasal, oral, etc. Injectables can be prepared in conventional forms, either as liquid solutions or suspension, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. If the agent to be test is a pharmaceutical composition or a drug, it may be administered with a suitable excipient (or vehicle). Suitable excipients include, for example, water, saline, aqueous dextrose, glycerol, ethanol or the like. Formulations for parenteral and nonparenteral drug delivery are known in the art and are set forth in Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing (1990). In testing for toxicity, the route of administration may be selected such that it is the same as the route of administration that will eventually be used in humans. For example, if

administration of penicillin is generally by injection, it may be advantageous to administer penicillin to a dog via injection, obtain tissues samples (*e.g.*, target tissue of the drug, if known), isolate cells and then obtain gene expression profiles. Once a series of gene expression profiles are compiled, cells may be used instead of whole animals and the gene expression profile of the cells, in response to an agent, may be compared to the compiled gene expression profiles of previous testing.

Methods of obtaining canine samples

[0086] Canine cells can be obtained from various sources. Different sources of cells can include, but are not limited to, biological samples such as tissue samples, blood, skin, biological fluids (*e.g.*, semen), and cell lines. Immortalized cell lines can be obtained from commercial sources, *e.g.*, Gibco BRL Life Sciences, or from non-commercial sources, *e.g.*, American Type Culture Collection (ATCC). One example of a cell line which is used in this invention is MDCK (ATCC accession number CCL-34) which is a canine kidney cell line. Other methods of obtaining canine cells include isolating cells obtained from tissue biopsies, blood, skin, or biological fluids. As is well known to one of skill in the art, isolating cells from tissue samples can be achieved using any variety of techniques. One example is to digest a tissue sample in an enzymatic solution to break up connective tissue and then agitate cells in the digested tissue to separate the cells from the connective tissue. Examples of other enzymes that can be used to digest tissue include neutral proteases, serine proteases including, but not limited to, trypsin, chymotrypsin, elastase, collagenase, and thermolysin. Another method is to homogenize the tissue sample or apply mechanical stress forces to the tissue sample to separate the cells from the basement membranes and allow the cells to become separated from within the tissue. In the alternative, DNA or RNA can be directly isolated from tissue samples, as exemplified in Example 1. Isolating cells from blood can be achieved by layering blood over a gradient (*e.g.*, Percoll™ or Ficoll™), spinning the blood-gradient layer in a centrifuge, and extracting the layer of cells from serum.

[0087] Sources from which cells are obtained can be any number of organs, including but not limited to liver, lung, heart, kidney, spleen, testes, thymus, and brain. In one embodiment, liver cells are used for toxicity studies where the agent to be administered is known or thought to induce liver malfunctions or liver toxicity. In other embodiments, when the target of the action delivered by the agent is known, the use of cells deriving from such target may yield more beneficial information regarding toxicological responses than if a tissue were selected at random. In another embodiment where the agent to be tested has unknown effects, a panel of cells isolated from different sources may be used. In the alternative, liver cells may be used in the absence of knowledge of the agent's target of action because the liver is known to process many toxins.

[0088] Canine cells obtained *ex vivo* or from a commercial or non-commercial source can cultured in media prior to being exposed to one or more agents. A wide variety of basal cell-sustaining media that can be used to keep the pH of the liquid in a range that promotes survival of canine cells. Non-limiting examples include F12/DMEM, Ham's F10 (Sigma), CMRL-1066, Minimal essential medium (MEM, Sigma), RPMI-1640 (Sigma), Dulbecco's Modified Eagle's Medium (DMEM, Sigma), and Iscove's Modified Eagle's Medium (IMEM). In addition, any of the basal nutrient media described in Ham and Wallace *Meth. Enz.*, **58**:44 (1979), Barnes and Sato *Anal. Biochem.*, **102**:255 (1980). In a preferred embodiment, Earle's Minimal Eagle's Medium (EMEM) supplemented with 10% fetal calf serum is used to culture canine cells. Cells can be grown in plates or in flasks. In a preferred embodiment, canine liver cells are grown in T-75 flasks contain Eagle's MEM supplemented with 10% fetal calf serum. Cells are grown and expanded to a level desired and needed for DNA or RNA isolation. Cells are removed from the plate or flask to isolate DNA or RNA. If the cells are adherent, trypsin or another equivalent may be used to release the cells from the plate or flask. Preferably at least about $1x10^2$ cells, more preferably at least about $1x10^3$ cells, more preferably at least about

$1x10^4$ cells, more preferably at least about $1x10^5$ cells, more preferably at least about $1x10^6$ cells, and even more preferably at least about $1x10^7$ cells are used as sources for DNA and RNA.

[0089] Nucleotide sequences from tissue samples are isolated using any number of commercially available kits *e.g.,* from Qiagen, GenHunter, Promega, etc. More detailed protocols on how to isolate DNA and/or RNA is disclosed in the Examples section. In general, a skilled artisan should take care to keep all reagents, tubes, and instruments sterile as to avoid contaminants which may affect how the results get interpreted. Once DNA or RNA has been isolated from cells which have been exposed to one or more agents, one or more toxicologically relevant genes are identified using the methods described above. The toxicologically relevant genes may be cloned into an expression vector, maintained in an expression vector or alternatively, the expression vector comprising the toxicologically relevant gene sequence may be transformed or transfected into a suitable host cell. Suitable host cells may be obtained from the ATCC or from commercial sources. Methods of isolating toxicologically relevant genes by cloning are further detailed in the Examples.

[0090] In some embodiments, the toxicologically relevant canine gene may be used to find a homologue in another animal, for example, in humans. The homologue may be then be used as a target for drug development or screening (*e.g.*, antigen for antibody development or cellular regulation).

[0091] In other embodiments, canine genes identified to be toxicologically relevant may be used to generate an array of toxicologically relevant canine genes. In this case, the gene may be cloned to facilitate the process of generating an array.

Methods of making an array

[0092] The isolated DNA or RNA is amplified to generate a product which can be attached to a substrate. In a preferred embodiment, the substrate is a solid substrate (*e.g.,* glass slide). The amplification process involves using primers which

have a reactive group (*e.g.*, amine group or derivative thereof) on one end of the primer, which is incorporated into the amplification product. One example of reactive primers that can be used is Amine Primers from Synthegen (Houston, TX; catalog #5002). The gene fragments which are attached to the glass slide can vary in length. The more nucleotides of a gene that are in the array, the tighter the binding and the greater the specificity in binding can occur. However, it is important to consider that longer fragments are more difficult to amplify and may contain point mutations or other errors associated with amplification. Therefore, the desired length of a gene or a fragment thereof that is to be included in the array should take into consideration the balance between a high specificity of binding obtained with a long (*e.g.*, >1 kb) gene sequence with the high mutational rate associated with a longer fragment. The gene fragments attached to the glass slide are at least about 50 base pairs (bp) in length, more preferably at least about 100 bp in length, more preferably at least about 200 bp, even more preferably at least about 300 bp, even more preferably at least about 400 bp, even more preferably at least about 500 bp in length. In a preferred embodiment, the gene fragments are about 500 bp in length. The region of a gene that is used to attach to a solid substrate to generate an array can be any portion of the gene, coding, non-coding, 5' end, 3' end, etc. In a preferred embodiment, about 500 base pairs of the 3' end of canine gene related to toxicological responses are selected to be included in an array.

[0093] In another embodiment, labeled antisense DNA probe may be made from amplified antisense RNA for hybridization to microarrays that contain sense targets. Exemplary protocols are disclosed in Examples 17 and 18. Methods of amplifying RNA are known in the art (see, for example Sambrook et al., *infra*) and methods of making microarrays with nucleic acids are disclosed herein.

[0094] Several techniques are well known to a skilled artisan for attaching a gene or a fragment thereof to a solid substrate such as a glass slide. One method is to attach an amine group, a derivative of an amine group, another group with a positive

charge or another group which is reactive to one end of a primer that is used to amplify a gene or a gene fragment to be included in the array. Subsequent amplification of a PCR product will then incorporate this reactive group onto one end of the product. The amplified product is then contacted with a solid substrate, such as a glass slide, which is coated with an aldehyde or another reactive group which will form a covalent link with the reactive group that is on the amplified PCR product and become covalently attached to the glass slide. Other methods using amino propryl silicane surface chemistry are disclosed by Corning Company at <http://www.cmt.corning.com.> other methods for making microarrays which are readily accessible at <http://cmgm.stanford.ecu/pbrownl>

[0095] In one embodiment of the invention, fluorescence-labeled single strand (or "first strand") cDNA probe is made from total or mRNA by first isolating RNA from control and treated cells, disclosed *supra*. This probe is hybridized to microarray slides spotted with DNA specific for toxicologically relevant genes. Methods for making the array and for labeling and making cDNA probes are disclosed in the Examples.

Method of using canine arrays to determine toxicological response

[0096] Once canine genes relevant to toxicological responses are identified, as disclosed *supra*, the genes or portions thereof are amplified and covalently attached to a substrate to produce an array as disclosed herein. In one embodiment, the substrate is a solid substrate including but not limited to glass slides, plastic slides, and metal chips. In a preferred embodiment, the solid substrate is a glass slide. Toxicological responses to agents are determined by isolating DNA or RNA from cells which have been exposed to one or more agents. The DNA or RNA is amplified and labeled (*e.g.*, fluorescent) as cDNA probes. The labeled cDNA probes are then used to hybridize with the microarray containing a collection of genes or fragments thereof ("target sequences") that are toxicologically relevant. The

differential expression of genes between exposed and unexposed provides information about a toxicological response.

[0097] By collecting many gene expression profiles from certain species, *e.g.*,, dogs, in response to one or more agents, a database can be built with collection of information about toxicological responses. With the database, it could be possible to predict toxicological response and/or stress response to specific agents or combinations thereof. The database can be stored on a computer and in a manner that allow for rapid searching when a comparison is desired. The database could store gene expression profiles for a particular toxin or alternatively, a group of toxins (*e.g.*, kidney-specific toxins). The database could also store gene expression profiles for a group of genes known to be affected by a particular toxin. When a gene expression profile is obtained, it may be compared with the gene expression profiles stored in the database to determine what type of organ is likely to be affect, or alternatively, which genes could also be associated with the toxic response. One or more genes could be analyzed in this manner as well as one or more toxins. The database may be stored in a form that allows for rapid access and analysis with compatible software programs.

[0098] The instant invention of canine gene arrays provides an alternative to testing on live canine animals. The canine gene array can provide answers on how a canine species might respond to a particular agent by examining at the differential gene expression associated with that particular agent in the array or comparison with a database of information collected from testing with a canine array. Further, canine gene arrays can provide answers about toxicological responses faster and more efficiently than testing *in vivo*.

[0099] The information generated from using canine gene arrays can be used to predict cellular and pathological responses as well as histological changes induced by exposure to agents. This is accomplished by analyzing the differential gene expression observed when canine gene arrays are used. Potential drugs or pharmaceutical compounds can be tested and data gathered for FDA approval in an

accelerated manner and can help pharmaceutical and biotechnology companies generate higher productivity with lower costs in research and development.

[0100] The canine gene array can also generate information that can be used to predict downstream effects, such as which pathways are affected by certain agents. This is accomplished by looking at the differential gene expression and analyzing which pathways contain the toxicological response genes and also which pathways the genes can affect. This information in turn can be used to predict tissue responses and ultimately whole organ responses. Examples of whole organ responses include but are not limited to organ functions, inflammatory responses, and autoimmune responses. Those of skill in the art can determine when the normal functions of an organ are compromised by exposure to one or more agents which are toxic. For example, a kidney's ability to filter toxins is compromised after an individual has been exposed to an agent. The ability to predict whole organ responses has great potential in the development of drugs, pharmaceutical compounds, and even in the use of chemicals.

[0101] The following Examples are provided to illustrate but not limit the present invention. It will be apparent to one of skill in the art that modifications can be made while keeping in the spirit and scope of the present invention.

EXAMPLES

Example 1 Isolation of total RNA From Animal Tissues

[0102] To isolate high quality and high purity total RNA from tissue samples, the following materials are used: Qiagen RNeasy midi kits, 2-mercaptoethanol, liquid N_2, tissue homogenizer, dry ice.

[0103] It is important to take precautions to minimize the risk of RNA degradation by RNase. Samples should be kept on ice when specified, gloves are worn at all times and work areas and equipment are treated with an RNase inhibitor, *e.g.*,, RNase Zap (Ambion® Products, Austin, TX). In order to prevent RNA degradation, it is highly preferable that the work area and materials used for this

procedure are clean and RNase-free. Autoclaving tips and microfuge tubes does not eliminate RNases. The following protocol is based on Qiagen® RNeasy® midi kit with modifications for optimal results. This total RNA isolation technique is used for RNA isolation from animal tissue and can be modified to accommodate smaller samples.

[0104] If tissue needs to be broken, it can be placed on a double layer of aluminum foil which is placed within a weigh boat containing a small amount of liquid nitrogen. The aluminum foil was placed around the tissue and then a blunt force was applied to the tissue with a small foil-wrapped hammer.

[0105] For liver or kidney, about 0.15-0.20 g of tissue was weighed and placed in a 15 ml conical tube. All tissue were kept on dry ice when other samples were being weighed.

[0106] About 3.8 ml of RLT buffer was added to the tube containing the sample. The RLT buffer® from Qiagen can be prepared beforehand by adding 10 µl betamercaptoethanol to each 1.0 ml of lysis buffer needed. The tissue was homogenized using the rotor-stator homogenizer for 45 seconds. A IKA Ultra Turrax T25 homogenizer set at speed 4 with the S25N-10G dispersing element can be used. Alternatively, a Virtishear Cyclone 750W rotor/stator homogenizer (Virtis item # 278077) can be used with the 7 mm microfine sawtooth shaft and generator (195 mm long with a processing range of 0.25 ml to 20 ml, item # 372718). After homogenization, samples were stored on ice until all samples were homogenized. To clean the homogenizing tip between samples, the tip was first run for a few seconds in 95 % ethanol and then rinsed by squirting with fresh 95% ethanol. This process was repeated with nanopure water.

[0107] The tissue lysate was centrifuged at room temperature for 10 minutes at 3700-3800 rpm in a Beckman GS-6 (or equivalent) centrifuge to remove nuclei thus reducing DNA contamination.

[0108] The supernatant of the lysate was transferred to a clean 15 ml conical tubes containing an equal volume of 70% EtOH in DEPC treated H_2O, being careful not to include any of the pellet or fatty layer and mixed. About 3.8 ml of sample was added to the RNeasy spin column placed in a 15 ml centrifuge tube and centrifuged at 3000 x g (3690-3710 rpm, Beckman GS-6) for 5 min. The flow-through was discarded. The remaining sample was added to the appropriate column and spun at 3000 x g for 5 minutes and the flow-through was discarded.

[0109] About 4.0 ml of Buffer RW1 (Qiagen®) was added to the column and spun as before then about 2.5 ml of buffer RPE (Qiagen®) was added to column and spun at 3000 x g (3690-3710 rpm, Beckman GS-6) for 2 minutes. In this example, RPE buffer was supplied as a concentrate so 4 volumes of 95% EtOH was added before use. For the midi kit, about 220 ml of 95% EtOH would be added to 55 ml of RPE. Another 2.5 ml of buffer RPE was added and spun for 5 minutes to also dry out column. The column, including the tip, should be dry for the next elution step.

[0110] For elution, the column that has the RNA bound to a clean 15 ml tube was transferred and 200 µl of RNase-free water was added to the column, allowed to sit for 1 minute, and spun for 3 minutes at 3000 x g (3690-3710 rpm, Beckman GS-6). This step was repeated into the same tube but with 200 µl RNase-free water.

[0111] To measure yield, the O.D. at 260 nm was taken and about 2.0 µl RNA was added to 98 µl H_2O. The following formula was used for calculations:

(Absorbance) x (dilution factor) x (40)/1000 = amount of RNA in µg/ml

For a sample calculation:

absorbance = 0.45

dilution factor = 50

$\frac{(0.45) \times 50 \times 40}{1000}$ = RNA concentration in µg/ml

[0112] This step is optional at this point in the procedure. It can be done after the LiCl precipitation step. The RNA solution was transferred to RNase-free 1.5 ml

Eppendorf-type tubes and about 1/3 volume (~300 µl if RNA was eluted in a total volume of 1000 µl) of LiCl precipitation solution (Ambion Cat. # 9480) was added. This mixture was placed at –20°C for about 30 minutes and spun at 14,000 rpm for 10 minutes. The mixture was decanted of the supernatant and the pellet was washed with 1.0 ml 70% ethanol and spun at 14,000 rpm for 10 minutes. The supernatant was decanted again and the pellet was allowed to dry to a certain degree (not complete dryness). The pellet was resuspended in RNA storage buffer (10 mM sodium citrate, Ambion Cat # 7000) starting with 300-400 µl and adding buffer as necessary until RNA is in solution. The RNA concentration was determined as disclosed using the RNA storage buffer as the blank. Samples were placed on ice until they were stored in the -80°C freezer.

Example 2 LiCl Precipitation

[0113] Isolated RNA samples can be precipitated using the following lithium chloride (LiCl) process either before or after measuring absorbance reading for quantitation purposes. The volume of the sample was measured. To this, about 1/3 volume of LiCl PPT Solution from Ambion (Cat # 9480) was added and mixed by inverting the tube. The LiCl should be in solution. If not, it may be necessary to adjust the pH to 8.0. The solution was placed at –20 ° C for 30 minutes and centrifuged at 4°C and 13,000 RPM for 10 minutes. If there is no visible pellet, it may help to return the sample to -20°C overnight and then repeat the centrifugation. The supernatant was transferred to a separate tube and washed by adding 1 ml of ice cold 70% ethanol in DEPC treated water and gently inverted. Then the tube was centrifuged at 4° C for 10 minutes and the supernatant was discarded and the pellet was air dried. The pellet was resuspended in RNA storage buffer (Ambion Cat # 7000). To determine the amount of buffer necessary, it was estimated that ~ 50% of the RNA was lost during this process. The RNA amount was quantitated spectrophotometrically.

Example 3 Isolation of total RNA from adherent cultured cells

[0114] Total RNA of high quality and high purity was isolated from cultured cells by using Qiagen RNeasy midi kits and 2-mercaptoethanol. RNA degradation by RNases is not desirable when synthesizing fluorescent cDNA for hybridization with the canine array. Precautions were taken to minimize the risk of RNA degradation by RNases by wearing gloves, treating work areas and equipment with an RNase inhibitor, for example, RNase Zap (Ambion® Products, Austin, TX) and keeping samples on ice. This total RNA isolation technique was based on a Qiagen® RNeasy® midi kit and was used with some modification for HepG2 (human hepatocyte) cells in T-75 flasks and maxi kit RNA isolation for cells in T-175 flasks.

[0115] Cells were checked under the microscope to make sure that they were viable. Cells were dosed with an agent, which could be a drug, chemical, or pharmaceutical composition, when they reached 60-80% confluence. It is preferable to avoid isolating RNA from flasks that have reached 100% confluence.

[0116] For adherent cells, media was discarded and flasks were washed with 1x cold PBS twice (20 ml then 10 ml for T-75 flasks; 40 ml then 20 ml for T-175 flasks). After the second PBS wash, the remaining PBS was removed with a pipette. Freshly prepared RLT buffer (RLT buffer requires the addition of 10 µl beta mercaptoethanol for each 1.0 ml RLT) was added directly to the cell culture flask. T-75 flasks received 3 ml RLT buffer and T-175 flasks received 5.0 ml RLT buffer. It is preferable to lightly agitate the flasks at this point. Flasks were lightly agitated to distribute the RLT buffer and the cells became a gelatinous layer. The cells were allowed to sit for 4 minutes, then fluid was withdrawn and placed in RNase-free tubes. An equivalent volume of 70% ethanol was added to each tube and vortexed to distribute evenly. If a precipitate with a string-like appearance forms, it is acceptable to remove and discard this string-like precipitate. The fluid was applied to a spin column and spun for 5 min at 3650 rpm in the Beckman GS-6 (or a similar centrifuge). The flow-through was discarded. About 4 or 15 ml (T-75 or T-175,

respectively) of RW1 buffer was applied and spun for 5 min at 3650 RPM. The flow through was discarded. About 2.5 ml RPE buffer (midi columns) or 10 ml RPE buffer (maxi columns) was applied and centrifuged for 3 minutes. The flow-through was discarded. Another 2.5 or 10 ml buffer RPE was applied and centrifuged for 5 minutes to dry out column before proceeding to the elution step. The column, including the tip, should be dry for the next step.

[0117] The column that has the RNA bound to it was transferred to a clean tube for elution. Then 150 µl of RNase-free water was added to midi columns and 500 µl of RNase-free water to columns, allowed to sit for 2-4 minutes and spun for 3 min at 3000 x g (3690-3710 rpm, Beckman GS-6 or a similar centrifuge). The elution was repeated with another 150µl or 500 µl of RNase-free water into the same tube. The elution was precipitated using the LiCl precipitation protocol, exemplified in Example 2, and resuspended in RNA storage buffer.

[0118] To measure yield, the O.D. reading was taken at 260 nm. About 2.0 µl RNA was added to 98 µl H2O and the O.D. reading was taken and calculated as follows:

(Absorbance) x (dilution factor) x (40)/1000 = amount of RNA in µg/ml

Example: absorbance = 0.45

dilution factor = 50

$\frac{(0.45) \times 50 \times 40}{1000}$ = RNA concentration in µg/ml

[0119] The yield should be between 200-400 µg of total RNA from a T-75 flask with greater than 50% confluency. The sample was stored in –80°C freezer.

Example 4 Identifying and isolating genes involved in toxicological responses

[0120] CANINE KIDNEY CELLS MDCK (ATCC ACCESSION NUMBER CCL-34) WERE DIVIDED INTO TWO ALIQUOTS. ONE GROUP WAS TREATED WITH CADMIUM CHLORIDE (SIGMA C-2544) AT THREE DIFFERENT CONCENTRATIONS OF 0.1 µM, 1 µM, AND 10 µM FOR 24

HOURS AND THE OTHER GROUP OF CELLS REMAINED UNTREATED FOR CONTROL PURPOSES. RNA WAS ISOLATED FROM BOTH GROUPS OF CELLS USING MESSAGECLEAN® KIT FROM GENHUNTER®. THE PROTOCOLS FROM THE MESSAGECLEAN® KIT WERE MODIFIED TO GENERATE MORE OPTIMAL CONDITIONS FOR REMOVING DNA CONTAMINATION. THEN, THESE INGREDIENTS WERE ADDED: 50 ML TOTAL RNA, 5.7 ML 10X REACTION BUFFER, 1.0 ML DNASE I (10 UNITS/ML) FOR A TOTAL VOLUME OF 56.7 ML. THE INGREDIENTS WERE MIXED WELL AND INCUBATED FOR 30 MINUTES AT 37° CELSIUS. THEN 40 ML PHENOL/CHLOROFORM MIXTURE (1:1 VOLUME) WAS ADDED AND THE MIXTURE WAS VORTEXED FOR 30 SECONDS AND ALLOWED TO SIT ON ICE FOR 10 MINUTES. THEN THE TUBE CONTAINING THE MIXTURE WAS SPUN IN AN EPPENDORF CENTRIFUGE AT 4 DEGREES FOR 5 MINUTES AT MAXIMUM SPEED. THE UPPER PHASE WAS COLLECTED, TRANSFERRED TO A NEW TUBE AND 5 ML OF 3M NAOAC AND 200 ML 95% ETHANOL WAS ADDED TO THE UPPER PHASE. THE MIXTURE WAS ALLOWED TO SIT FOR AT LEAST ONE HOUR AT -80° C AND THEN SPUN FOR ABOUT 10 MINUTES AT 4° C. THE SUPERNATANT WAS REMOVED AND THE RNA DRIED FOR A FEW MINUTES. SUBSEQUENTLY, THE RNA WAS SUSPENDED IN 11 ML DEPC H_20. 1 ML WAS USED TO MEASURE $A_{260/280}$ IN 50 ML H_20. THE RNA WAS STORED AS 1-2 MG ALIQUOTS AT -80°C. IMMEDIATELY PRIOR TO DIFFERENTIAL DISPLAY, THE APPROPRIATE AMOUNT OF RNA WAS DILUTED TO 0.1 MG/ML WITH DEPC H_20 . IT IS IMPORTANT TO AVOID USING THE DILUTED RNA AFTER FREEZE-THAW CYCLE.

[0121] RNAIMAGE® KITS WERE USED AND PROTOCOLS FROM THE RNAIMAGE® KITS WERE ALTERED TO OPTIMIZE MORE SUCCESSFUL

MRNA DIFFERENTIAL DISPLAY. THE FOLLOWING SECTION DESCRIBES THE METHOD BY WHICH THIS WAS ACCOMPLISHED:

Reverse transcription

[0122] IN A TUBE, THE FOLLOWING INGREDIENTS WERE ADDED: 9.4 ML DH_20, 4.0 ML 5X RT BUFFER, 1.6 ML DNTP (250 MM), 2.0 ML OF 0.1 MG/ML FRESHLY DILUTED TOTAL RNA THAT WAS DNASE-FREE, 2.0 ML H-T_{11}M (2 MM) FOR A TOTAL VOLUME OF 19 ML. THE INGREDIENTS WERE MIXED WELL AND INCUBATED AT 65°C FOR 5 MINUTES, 37°C FOR 60 MINUTES, 75°C FOR 5 MINUTES, AND HELD AT 4°C. AFTER THE TUBES HAD BEEN AT 37°C FOR 10 MINUTES, AND 1 ML OF SUPERSCRIPT II REVERSE TRANSCRIPTASE (LIFE TECHNOLOGIES INC.) WAS ADDED TO EACH REACTION, AND QUICKLY MIXED BY FINGER TAPPING THE TUBES BEFORE THE INCUBATION CONTINUED. AT THE END OF THE REVERSE TRANSCRIPTION, THE TUBES WERE SPUN BRIEFLY TO COLLECT CONDENSATION. THE TUBES WERE SET ON ICE FOR PCR OR STORED AT -20°C FOR LATER USE.

PCR

[0123] THE FOLLOWING INGREDIENTS WERE USED FOR A PCR REACTION: 10 ML DH_20, 2 ML 10X PCR BUFFER, 1.6 ML DNTP (25 MM), 2 ML OF 2 MM H-AP PRIMER, 2 ML OF 2 MM H-T_{11}M, 2 ML RT-MIX DESCRIBED ABOVE (MUST CONTAIN THE SAME H-T_{11}M USED FOR PCR), 0.2 ML α-^{33}P DATP (2000 CI/MMOLE), 0.2 ML TAQ DNA POLYMERASE FROM PE BIOSYSTEMS FOR A TOTAL VOLUME OF 20 ML. THE TUBE CONTAINING ALL THESE INGREDIENTS WERE MIXED WELL BY PIPETING UP AND DOWN AND PLACED IN A THERMOCYCLER AT 95°C FOR 5 MINUTES AND THEN AMPLIFIED FOR 40 CYCLES UNDER THE CONDITIONS OF 94°C FOR 30 SECONDS, 40°C FOR 2 MINUTES, 72°C FOR 30

SECONDS AND FINALLY HELD AT 4°C UNTIL THE SAMPLES ARE REMOVED FROM THE THERMOCYCLER.

Gel electrophoresis

[0124] A 6% DENATURING POLYACRYLAMIDE GEL IN TBE WAS PREPARED AND ALLOWED TO POLYMERIZE FOR AT LEAST 2 HOURS BEFORE USING. THEN THE GEL WAS RUN FOR ABOUT 30 MINUTES BEFORE ANY SAMPLES WERE LOADED. IT IS IMPORTANT FOR ALL THE SAMPLE WELLS IN THE GEL TO BE FLUSHED AND CLEARED OF ALL UREA PRIOR TO LOADING ANY SAMPLES IN THE WELLS. ABOUT 3.5 ML OF EACH SAMPLE WAS MIXED WITH 2 ML OF LOADING DYE AND INCUBATED AT 80°C FOR 2 MINUTES IMMEDIATELY BEFORE LOADING ONTO THE 6% GEL. IN THIS EXAMPLE, THE LOADING DYE WAS XYLENE AND AFTER THE GEL WAS LOADED WITH THE SAMPLES OBTAINED FROM THE ROUNDS OF PCR, THE GEL WAS RUN AT 60 WATTS OF CONSTANT POWER UNTIL THE XYLENE DYE WAS ABOUT 6 INCHES FROM THE BOTTOM OF THE GEL. ONCE THE POWER WAS TURNED OFF, THE GEL WAS BLOTTED ONTO A LARGE SHEET OF EXPOSED AUTORADIOGRAPH FILM. THE GEL WAS COVERED WITH PLASTIC WRAP AND UNDER DARK CONDITIONS, THE GEL WAS PLACED IN A LARGE AUTORADIOGRAPH CASSETTE WITH A NEW SHEET OF UNEXPOSED FILM, MARKED FOR ORIENTATION, AND THE FILM WAS ALLOWED TO BE EXPOSED TO THE GEL AT -80°C. THE EXPOSURE PERIOD CAN BE ANYWHERE FROM OVERNIGHT TO 72 HOURS. ONCE THE FILM HAS BEEN DEVELOPED, BANDS OF INTEREST WERE IDENTIFIED BY ALIGNMENT WITH THE DEVELOPED FILM AND SUBSEQUENTLY ISOLATED BY CUTTING THE BAND OF INTEREST OUT OF THE POLYACRYLAMIDE GEL WITH A CLEAN SCALPEL BLADE. THE

ISOLATED BAND WAS PLACED IN 100 ML OF WATER AND BOILED AT 95% FOR 5 MINUTES.

PCR to amplify gel band

[0125] PCR WAS SET UP TO AMPLIFY THE GEL BAND. THE RE-AMPLIFICATION SHOULD BE DONE USING THE SAME PRIMER SET AND PCR CONDITIONS EXCEPT THE DNTP CONCENTRATIONS SHOULD BE AT 20 MM. THE FOLLOWING INGREDIENTS WERE COMBINED FOR THE PCR REACTION: 20.4 ML H_20, 4 ML 10X PCR BUFFER, 3.2 ML OF 250 MM DNTPS , 4 ML OF 2 MM H-AP PRIMERS, 4 ML OF 2 MM H-T_{11}M, 4 ML TEMPLATE (OUT OF THE 100 ML CONTAINING GEL BAND), AND 0.5 ML TAQ POLYMERASE FOR A TOTAL VOLUME OF 40 ML. THESE INGREDIENTS WERE HEATED TO 95°C FOR 5 MINUTES AND THEN CYCLED FOR 40 CYCLES UNDER THE CONDITIONS OF 94°C FOR 30 SECONDS, 40°C FOR 2 MINUTES, 72°C FOR 30 SECONDS FOLLOWED BY A FINAL EXTENSION AT 72°C FOR 5 MINUTES AND FINALLY HELD AT 4°C UNTIL THE SAMPLES ARE REMOVED FROM THE THERMOCYCLER. ABOUT 4 ML OF THE PCR REACTION WAS REMOVED AND RUN ON A 1% AGAROSE GEL TO ASCERTAIN THE SUCCESS OF THE PCR REACTION.

Cloning amplified fragments

[0126] TO CLONE THE AMPLIFIED FRAGMENTS, PRODUCTS FROM DIFFERENT SOURCES (*E.G.*, GENHUNTER OR INVITROGEN) MAY BE USED TO ACHIEVE THE DESIRED CLONED PRODUCT. IN THIS EXAMPLE, INVITROGEN'S TOPO TA CLONING KIT® WAS USED AND THE FOLLOWING MATERIAL WAS COMBINED IN A REACTION TUBE: 2 ML OF FRESHLY RUN PCR PRODUCT, 2 ML OF STERILE H_20, 1 ML OF PCR-TOPO VECTOR FOR A FINAL VOLUME OF 5 ML. THE COMBINED INGREDIENTS WERE MIXED GENTLY AND INCUBATED FOR 5 MINUTES AT ROOM TEMPERATURE. THEN 1 ML OF 6X TOPO CLONING STOP SOLUTION WAS

ADDED AND ALL COMBINED INGREDIENTS WERE MIXED FOR ABOUT 10 SECONDS AT ROOM TEMPERATURE AND THEN SET ON ICE. ONE SHOT™ CELLS WERE THAWED ON ICE. 2 ML OF THE TOPO CLONING REACTION WAS ADDED TO THE ONE SHOT™ CELLS, MIXED, AND INCUBATED ON ICE FOR 30 MINUTES. THE CELLS WERE HEAT SHOCKED AT 42°C FOR 30 SECONDS WITHOUT SHAKING AND INCUBATED ON ICE FOR 2 MINUTES. THEN 250 ML OF ROOM TEMPERATURE SOC WAS ADDED TO THE HEAT SHOCKED CELLS AND MIXED. THE CELLS WERE THEN PLACED AT 37°C FOR 30 MINUTES. ABOUT 50-100 ML OF THE CELLS WERE SPREAD ON 2 XYT PLATES CONTAINING 100 MG/ML AMPICILLIN AND X-GAL. THE PLATES WERE INCUBATED OVERNIGHT AT 37°C AND THE NEXT MORNING, 3 WHITE COLONIES WERE SELECTED FOR ANALYSIS.

Screening colonies for correct recombinant plasmids

[0127] PCR WAS USED TO ASCERTAIN WHETHER THE WHITE COLONIES SELECTED CONTAINED THE CORRECT RECOMBINANT PLASMID. THE FOLLOWING INGREDIENTS WERE COMBINED FOR THE PCR REACTION: 21 ML H_20, 2.5 ML 10X PCR BUFFER, 0.12 ML OF 10 MM DNTPS, 1 ML OF 25 NG/ML T7 PRIMER, 1 ML GENE SPECIFIC LEFT OR RIGHT PRIMER AT 25 NG/ML, TEMPLATE (A TOOTHPICK WAS USED TO TRANSFER COLONY FROM TRANSFORMATION PLATE TO TUBE BY SWISHING THE TOOTHPICK AROUND IN THE REACTION MIX), AND 0.5 ML TAQ POLYMERASE FOR A TOTAL VOLUME OF 25 ML. THE REACTION MIX WAS RUN AT 95°C FOR 5 MINUTES AND THEN CYCLED 35 TIMES UNDER THE CONDITIONS OF 95° C FOR 30 SECONDS, 45°C FOR 30 SECONDS, 72° C FOR 30 SECONDS, AND FOLLOWED BY 72° C FOR 5 MINUTES AND FINALLY 4°C UNTIL SAMPLES ARE REMOVED FROM THE THERMOCYCLER. ABOUT 4 ML OF THE PCR PRODUCT WAS REMOVED

AND RUN ON A 1% AGAROSE GEL TO ASCERTAIN THE SUCCESS OF THE PCR REACTION. BACTERIAL COLONIES CORRESPONDING TO THE COLONIES WHICH YIELDED POSITIVE PCR RESULTS WERE GROWN OVERNIGHT IN LB MEDIA CONTAINING 100 MG/ML AMPICILLIN AT 37° C WITH CONSTANT SHAKING. PLASMID DNA WERE ISOLATED FROM THE OVERNIGHT CULTURES AND SEQUENCED USING A T7 PRIMER. SEQUENCES WERE THEN COMPARED TO SEQUENCES IN THE GENBANK DATABASE TO CONFIRM THAT THE CORRECT GENE FRAGMENT WAS CLONED. GENE FRAGMENTS WERE THEN AMPLIFIED BY PCR FROM THE PLASMID DNA. THE UNINCORPORATED PRIMERS AND DNTPS WERE REMOVED AND THE RESULTING GENE FRAGMENTS WERE ARRAYED ON GLASS SLIDES FOR THE PURPOSES OF MEASURING DIFFERENTIAL GENE EXPRESSION USING THE PHASE-1 MOLECULAR TOXICOLOGY MICROARRAY PRODUCTS. TABLE 5 INDICATES TOXICOLOGICALLY RELEVANT GENES WHICH HAVE BEEN IDENTIFIED USING METHODS DISCLOSED IN THIS EXAMPLE.

Example 5 Identifying and isolating toxicologically relevant genes from canine databases

[0128] ONE METHOD THAT WAS USED TO IDENTIFY AND ISOLATE TOXICOLOGICALLY RELEVANT GENES FOR INCLUSION IN A CANINE ARRAY WAS TO SEARCH A PUBLIC DATABASE (*E.G.*,, GENBANK) FOR TOXICOLOGICALLY RELEVANT CANINE GENES. ONCE THESE GENES WERE IDENTIFIED, PRIMERS WERE OBTAINED AND USED IN AN AMPLIFICATION PROCESS WITH CDNA LIBRARY MADE FROM CANINE CELLS. AS DISCLOSED HEREIN, CDNA LIBRARY CAN BE MADE FROM A VARIETY OF SOURCES. IN THIS EXAMPLE, THE CDNA LIBRARY WAS MADE FROM BEAGLE LIVER CELLS. THE AMPLIFIED PRODUCT WAS CLONED INTO AN EXPRESSION VECTOR AND SEQUENCED TO CONFIRM

THAT THE SEQUENCE MATCHED OR WAS SUBSTANTIALLY SIMILAR TO THE GENE SEQUENCE INFORMATION OBTAINED FROM GENBANK. CONFIRMED AMPLIFIED GENE PRODUCTS WERE THEN INCORPORATED INTO A CANINE ARRAY USING THE METHODS DISCLOSED HEREIN TO IMMOBILIZE THE GENE PRODUCT, OR TARGET SEQUENCE, TO A GLASS SLIDE. TOXICOLOGICALLY RELEVANT GENES WHICH HAVE BEEN IDENTIFIED AND ISOLATED IN THIS MANNER ARE LISTED IN TABLE 1.

Example 6 Identifying and isolating toxicologically relevant genes from human databases

[0129] ONE METHOD THAT WAS USED TO IDENTIFY AND ISOLATE TOXICOLOGICALLY RELEVANT GENES FOR INCLUSION IN A CANINE ARRAY WAS TO SEARCH A PUBLIC DATABASE (*E.G.*,, GENBANK) FOR TOXICOLOGICALLY RELEVANT HUMAN GENES. ONCE THESE GENES WERE IDENTIFIED, PRIMERS WERE OBTAINED AND USED IN AN AMPLIFICATION PROCESS WITH CDNA LIBRARY MADE FROM CANINE CELLS. AS DISCLOSED HEREIN, CDNA LIBRARY CAN BE MADE FROM A VARIETY OF SOURCES. IN THIS EXAMPLE, THE CDNA LIBRARY WAS MADE FROM BEAGLE LIVER CELLS. THE AMPLIFIED PRODUCT WAS CLONED INTO AN EXPRESSION VECTOR AND SEQUENCED TO CONFIRM THAT THE SEQUENCE MATCHED OR WAS SUBSTANTIALLY SIMILAR TO THE GENE SEQUENCE INFORMATION OBTAINED FROM GENBANK. CONFIRMED AMPLIFIED GENE PRODUCTS WERE THEN INCORPORATED INTO A CANINE ARRAY USING THE METHODS DISCLOSED HEREIN TO IMMOBILIZE THE GENE PRODUCT, OR TARGET SEQUENCE, TO A GLASS SLIDE. TOXICOLOGICALLY RELEVANT GENES WHICH HAVE BEEN IDENTIFIED AND ISOLATED IN THIS MANNER ARE LISTED IN TABLE 6.

Example 7 Identifying and isolating toxicologically relevant genes using *de novo* primers

[0130] ANOTHER METHOD WAS USED TO IDENTIFY AND ISOLATE TOXICOLOGICALLY RELEVANT GENES. TOXICOLOGICALLY RELEVANT GENES WERE IDENTIFIED USING A PUBLIC DATABASE (*E.G.*,, GENBANK) AND SEQUENCES CORRESPONDING WITHIN THESE GENES WERE SYNTHESIZED *DE NOVO* AND USED IN AMPLIFICATION REACTIONS. THE AMPLIFIED PRODUCT WAS CLONED INTO AN EXPRESSION VECTOR AND SEQUENCED TO CONFIRM THAT THE SEQUENCE MATCHED OR WAS SUBSTANTIALLY SIMILAR TO THE GENE SEQUENCE INFORMATION OBTAINED FROM GENBANK. CONFIRMED AMPLIFIED GENE PRODUCTS WERE THEN INCORPORATED INTO A CANINE ARRAY USING THE METHODS DISCLOSED HEREIN TO IMMOBILIZE THE GENE PRODUCT, OR TARGET SEQUENCE, TO A GLASS SLIDE. TOXICOLOGICALLY RELEVANT GENES WHICH HAVE BEEN IDENTIFIED AND ISOLATED IN THIS MANNER ARE LISTED IN TABLE 3.

[0131]

Example 8 Attaching toxicologically relevant genes to glass slides

[0132] THE GENES TO BE ATTACHED TO THE GLASS SLIDES CAN BE AMPLIFIED AS PROVIDED HEREIN. AN IMPORTANT MODIFICATION TO THE AMPLIFICATION PROCESS WAS THE INCLUSION OF AMINE PRIMERS, WHICH CAN BE OBTAINED FROM ANY COMMERCIAL SOURCE, *E.G.*, SYNTHEGEN, SUCH THAT A REACTIVE AMINE GROUP, A DERIVATIVE THEREOF, OR ANOTHER REACTIVE GROUP WAS INCLUDED IN THE AMPLIFIED PRODUCT. THE AMPLIFIED PRODUCT WAS PURIFIED BY ANY NUMBER OF METHODS DISCLOSED HEREIN AND IMMOBILIZED OR "SPOTTED" ONTO A SOLID SUBSTRATE, SUCH AS A GLASS SLIDE, WHICH CAN

REACT WITH THE AMINE GROUP ON THE AMPLIFIED PRODUCT AND FORM A COVALENT LINKAGE.

MD Array Spotter Operation

[0133] The terminology and equipment used in this example comprised the following:

Spotter: MD Generation II Array Spotter main instrument

Spotting Chamber: Area of spotter enclosed in glass which houses the pins, plates, trays and most spotter machinery.

Controller: Dedicated Dell Computer and Monitor to right of Spotter Unit

Pins: (6) fine tubes in the Spotter Unit which pick-up and spot the Target

Slides: Std. size glass microscope slides with a special coating on one side

Plates: Plastic 96 well plates which hold the Target solution to be spotted

Target: A solution of PCR product which the spotter deposits on the slides.

N2 Tank: 5 ft. high steel gas tank labeled "Nitrogen, Compressed"

N2 : The N2 gas from the N2 tank

Air Conditioner: Kenmore air conditioner installed in window of spotting chamber

Humidifier 1: Essick 2000 Evaporative Cooler against the window

Humidifier 2: Bemis Airflow with white flexible duck into the Spotter Unit

Humidifier 3: Bemis Airflow against the wall

Humidifier 4: Kenmore QuietComfort 7

Vacuum Pump: Gast Laboratory Oilless Piston Vacuum Pump

Dampbox: The plastic sealable container containing an NaCl / water slurry

[0134] Materials used for reagent solutions were: Nanopure water, 0.2 M KCl (1/10 dilution of Stock 2M KCL in water), and 95% EtOH Reagent. The temperature control was adjusted to 60°. The spotter chambers were adjusted to be greater than 39 % relative humidity and less than 65° C. The spotting pins were pre-washed for 20 cycles.

Slide Preparation/Loading:

[0135] When the pre-wash was completed, the slides were first each blown with N2 gas for about 2 seconds per side. The slides were inserted into the Spotter following Array Spotter Run Values. The slides were aligned using a clean narrow rod orienting it on the center right edge of the slide and gently pushed to the left until the slide was aligned vertically against the metal pins. After slides were loaded and straightened, a visual check was done to make sure no more debris had fallen. The humidity was confirmed to be greater than 39% relative humidity. The MD spotter recognizes 16 plates as a maximum for a run and will pause automatically after 8 plates. The MD spotter also advances sequentially to plates in an invariable order and is not programmable to accommodate unique plate sourcing scheme. Therefore, it was important to manually rotate (or shuffle) plates to accomplish the spotting for the canine arrays.

BLOCKING (SLIDE PREPARATION POST-SPOTTING)

[0136] THIS BLOCKING PROCEDURE IS IMPORTANT BECAUSE IT REDUCES THE NON-SPECIFIC BACKGROUND SIGNALS. THE AMOUNTS PROVIDED IN THIS PROTOCOL ARE FOR 19 SLIDES, HOWEVER, A SKILLED ARTISAN MAY MAKE MODIFICATIONS ACCORDINGLY. MORE STAINING DISHES AND SLIDE RACKS WILL BE REQUIRED IF MORE THAN 19 SLIDES ARE TO BE BLOCKED. A CLEAN GLASS CONTAINER WAS OBTAINED AND FILLED WITH

NANOPURE H20. THE CONTAINER WAS PLACED ON A HOT PLATE AND HEATED TO A HIGH TEMPERATURE. A BLOCKING SOLUTION WAS MADE BY ADDING 2.5 ML OF 20% SDS TO 500 ML BLOCKING SOLUTION BOTTLE. THE BLOCKING SOLUTION WAS WARMED IN MICROWAVE FOR 2.5 MINUTES AND CHECKED TO DETERMINE IF THE TEMPERATURE HAD REACHED 50°C. IF THE TEMPERATURE OF THE SOLUTION WAS NOT AT YET 50°C, THEN THE SOLUTION WAS WARMED IN THE MICROWAVE AT 10 SECOND INTERVALS UNTIL IT REACHED THE DESIRED TEMPERATURE. ONE STAINING DISH WAS PLACED ON AN ORBITAL SHAKER WITH 4X SSC SOLUTION AND TURNED TO AN AGITATION SPEED OF 75 RPM. SLIDES WERE PLACED IN METAL RACKS AND PLACED IN BOILING WATER FOR SEVERAL MINUTES (*E.G.*, 2 MINUTES). THE SLIDES WERE TAKEN OUT OF BOILING WATER AND ALLOWED TO COOL BRIEFLY. THE SLIDES WERE THEN TRANSFERRED TO STAINING CONTAINER CONTAINING 4X SSC SOLUTION ON ORBITAL SHAKER FOR SEVERAL MINUTES (*E.G.*, 2 MINUTES), RINSED WITH NANOPURE WATER IN A STAINING CONTAINER, AND THEN BRIEFLY PLACED IN BLOCKING SOLUTION FOR ABOUT 15 MINUTES. AFTER 15 MINUTES, THE SLIDES WERE TAKEN OUT OF THE BLOCKING SOLUTION AND RINSED THREE TIMES BY DIPPING INTO THREE SEPARATE CONTAINERS WITH NANOPURE WATER EACH TIME. THE TOPS OF THE SLIDES WERE DABBED LIGHTLY WITH A TISSUE AND THE SLIDES WERE PLACED IN A CENTRIFUGE FOR ABOUT 5 MINUTES AT A SPEED OF 1000 RPM.

Example 9 Microarray RT Reaction

[0137] Fluorescence-labeled first strand cDNA probe was made from total or mRNA by first isolating RNA from control and treated cells, disclosed *supra*. This

probe is hybridized to microarray slides spotted with DNA specific for toxicologically relevant genes. The materials needed to practice this example are: total or messenger RNA, primer, Superscript II buffer, dithiothreitol (DTT), nucleotide mix, Cy3 or Cy5 dye, Superscript II (RT), ammonium acetate, 70% EtOH, PCR machine, and ice.

[0138] The volume of each sample that would contain 20µg of total RNA (or 2µg of mRNA) was calculated. The amount of DEPC water needed to bring the total volume of each RNA sample to 14 µl was also calculated. If RNA is too dilute, the samples are concentrated to a volume of less than 14 µl in a speedvac without heat. The speedvac must be capable of generating a vacuum of 0 Milli-Torr so that samples can freeze dry under these conditions. Sufficient volume of DEPC water was added to bring the total volume of each RNA sample to 14 µl. Each PCR tube was labeled with the name of the sample or control reaction. The appropriate volume of DEPC water and 8 µl of anchored oligo dT mix (stored at -20°C) was added to each tube.

[0139] Then the appropriate volume of each RNA sample was added to the labeled PCR tube. The samples were mixed by pipeting. The tubes were kept on ice until all samples are ready for the next step. It is preferable for the tubes to kept on ice until the next step is ready to proceed. The samples were incubated in a PCR machine for 10 minutes at 70°C followed by 4°C incubation period until the sample tubes were ready to be retrieved. The sample tubes were left at 4°C for at least 2 minutes.

[0140] The Cy dyes are light sensitive, so any solutions or samples containing Cy-dyes should be kept out of light as much as possible (*e.g.*, cover with foil) after this point in the process. Sufficient amounts of Cy3 and Cy5 reverse transcription mix were prepared for one to two more reactions than would actually be run by scaling up the following recipes:

[0141]

For labeling with Cy3

8 ul 5x First Strand Buffer for Superscript II

4 ul 0.1 M DTT

2 ul Nucleotide Mix

2 ul of 1:8 dilution of Cy3 (e.g.,, 0.125mM Cy3dCTP).

2 ul Superscript II

For labeling with Cy5

8 ul 5x First Strand Buffer for Superscript II

4 ul 0.1 M DTT

2 ul Nucleotide Mix

2 ul of 1:10 dilution of Cy5 (e.g.,, 0.1mM Cy5dCTP).

2 ul Superscript II

[0142] About 18 µl of the pink Cy3 mix was added to each treated sample and 18 µl of the blue Cy5 mix was added to each control sample. Each sample was mixed by pipeting. The samples were placed in a PCR machine for 2 hours at 45°C followed by 4°C until the sample tubes were ready to be retrieved. The samples were transferred to Eppendorf tubes containing 600 µl of ethanol precipitation mixture. Some of the EtOH precipitation mixture was used to rinse the PCR tubes. The tubes were inverted to mix. Samples were placed in –80°C freezer for at least 20-30 minutes. If desired, samples may be left at -20°C overnight or over the weekend.

[0143] The samples were centrifuged for 15 minutes at 20800 x g (14000 rpm in Eppendorf model 5417C) and carefully the supernatant was decanted. A visible pellet was seen (pink/red for Cy3, blue for Cy5). It is a preferable to centrifuge the tubes at a fixed position so the pellet will be at a known area in the tube. In some rare instances, the probe is seen spread on one side of the tube instead of a tight pellet. If the pellet is white or nonexistent, the reaction has not occurred to maximal efficiency.

[0144] Ice cold 70% EtOH (about 1 ml per tube) was used to wash the tubes and the tubes were subsequently inverted to clean tube and pellet. The tubes were centrifuged for 10 minutes at 20800 x g (14000 rpm in Eppendorf model 5417C), then the supernatant was carefully decanted. The tubes were flash spun and any remaining EtOH was removed with a pipet. The tubes were air dried for about 5 to 10 minutes. protected from light. The length of drying time will depend on the natural humidity of the environment. For example, an environment in Santa Fe would require about 2 to 5 minutes of drying time. It is preferable that the pellet are not overdried.

[0145] When the pellets were dried, they are resuspended in 80 ul nanopure water. The cDNA/mRNA hybrid was denatured by heating for 5 minutes at 95°C in a heat block and flash spun.

Example 10 Purification of Cy –Dye Labeled cDNA

[0146] To purify fluorescence-labeled first strand cDNA probes, the following materials were used: Millipore MAHV N45 96 well plate, v-bottom 96 well plate (Costar), Wizard DNA binding Resin, wide orifice pipette tips for 200 to 300 µl volumes, isopropanol, nanopure water. It is highly preferable to keep the plates aligned at all times during centrifugation. Misaligned plates can lead to sample cross contamination and/or sample loss. It is also important that plate carriers are seated properly in the centrifuge rotor.

[0147] The lid of a "Millipore MAHV N45" 96 well plate was labeled with the appropriate sample numbers. A blue gasket and waste plate (v-bottom 96 well) was attached. Wizard DNA Binding Resin (Promega catalog #A1151) was shaken immediately prior to use for thorough resuspension. About 160 µl of Wizard DNA Binding Resin was added to each well of the filter plate that was used. If this was done with a multi-channel pipette, wide orifice pipette tips would have been used to prevent clogging. It is highly preferable not to touch or puncture the membrane of the filter plate with a pipette tip. Probes were added to the appropriate wells (80 µl

cDNA samples) containing the Binding Resin. The reaction is mixed by pipeting up and down ~10 times. It is preferable to use regular, unfiltered pipette tips for this step. The plates were centrifuged at 2500 rpm for 5 minutes (Beckman GS-6 or equivalent) and then the filtrate was decanted. About 200 µl of 80% isopropanol was added, the plates were spun for 5 minutes at 2500 rpm, and the filtrate was discarded. Then the 80% isopropanol wash and spin step was repeated. The filter plate was placed on a clean collection plate (v-bottom 96 well) and 80 µl of Nanopure water, pH 8.0-8.5 was added. The pH was adjusted with NaOH. The filter plate was secured to the collection plate with tape to ensure that the plate did not slide during the final spin. The plate sat for 5 minutes and was centrifuged for 7 minutes at 2500 rpm. If there are replicates of samples they should be pooled.

Example 11 Fluorescence Readings of cDNA Probe

[0148] To semi-quantitatively assess the incorporation of fluorescence into cDNA probes and to concentrate probes prior to hybridization, the following material was used: 384 well, 100 µl assay plate (Falcon Microtest catalog #35-3980) and Wallac Victor 1420 Multilabel counter (or equivalent).

[0149] It is preferable that a consistent amount of cDNA is pipeted into the 384-well plate wells because readings will vary with volume. Controls or identical samples should be pooled at this step, if required. The probes were transferred from the Millipore 96 well plate to every other well of a 384 well assay plate (Falcon Microtest). This was done using a multi-channel pipette. For replicate samples that have been pooled, 60 µl aliquots were transferred into wells of the assay plate.

[0150] The Cy-3 and Cy-5 fluorescence was analyzed using the Wallac 1420 workstation programmed for reading Cy3-Cy-5 in the 384-well format and the data was saved to disk. The typical range for Cy-3 (20µg) is 250-700,000 fluorescence units. The typical range for Cy-5 (20µg) is 100-250,000 fluorescence units. Settings for the Wallac 1420 fluorescence analyzer were as follows:

Cy3

CW lamp energy = 30445

Lamp filter = P550 slot B3

Emission filter= D572 dysprosium slot A4

Emission aperture = normal

Count time = 0.1 s

Cy5

CW lamp energy = 30445

Lamp filter = D642 samarium slot B7

Emission filter= D670 slot A8

Emission aperture = normal

Count time = 0.1 s

[0151] Dry-down Process

[0152] Concentration of the cDNA probes is highly preferable so that they can be resuspended in hybridization buffer at the appropriate volume. The volume of the control cDNA (Cy-5) was measured and divide by the number of samples to determine the appropriate amount to add to each test cDNA (Cy-3). Eppendorf tubes were labeled for each test sample and the appropriate amount of control cDNA was allocated into each tube. The test samples (Cy-3) were added to the appropriate tubes. These tubes were placed in a speed-vac to dry down, with foil covering any windows on the speed vac. At this point, heat (45°C) may be used to expedite the drying process. Time will vary depending on the machinery. The drying process takes about one hour for 150 µl samples dried in the Savant. Samples may be saved in dried form at -20°C for up to 14 days.

Example 12 Microarray Hybridization

[0153] To hybridize labeled cDNA probes to single stranded, covalently bound DNA target genes on glass slide microarrays, the following material were used:

formamide, SSC, SDS, 2 μm syringe filter, salmon sperm DNA, hybridization chambers, incubator, coverslips, parafilm, heat blocks. It is preferable that the array is completely covered to ensure proper hybridization.

[0154] About 30 μl of hybridization buffer was prepared per sample. Slightly more than is what is needed should be made since about 100 μl can be lost during filtration.

Hybridization Buffer:	for 100 μl:
50% Formamide	50 μl formamide
5X SSC	25 μl 20X SSC
0.1% SDS	25 μl 0.4% SDS

[0155] The solution was filtered through 0.2 μm syringe filter, then the volume was measured. About 1 μl of salmon sperm DNA (10mg/ml) was added per 100 μl of buffer. Materials used for hybridization were: 2 Eppendorf tube racks, hybridization chambers (2 arrays per chamber), slides, coverslips, and parafilm. About 30 μl of nanopure water was added to each hybridization chamber. Slides and coverslips were cleaned using N_2 stream. About 30 μl of hybridization buffer was added to dried probe and vortexed gently for 5 seconds. The probe remained in the dark for 10-15 minutes at room temperature and then was gently vortexed for several seconds and then was flash spun in the microfuge. The probes were boiled for 5 minutes and centrifuged for 3 min at 20800 x g (14000 rpm, Eppendorf model 5417C). Probes were placed in 70 °C heat block. Each probe remained in this heat block until it was ready for hybridization.

[0156] Pipette 25 μl onto a coverslip. It is highly preferable to avoid the material at the bottom of the tube and to avoid generating air bubbles. This may mean leaving about 1 μl remaining in the pipette tip . The slide was gently lowered, face side down, onto the sample so that the coverslip covered that portion of the slide containing the array. Slides were placed in a hybridization chamber (2 per chamber).

The lid of the chamber was wrapped with parafilm and the slides were placed in a 42°C humidity chamber in a 42°C incubator . It is preferable to not let probes or slides sit at room temperature for long periods. The slides were incubated for 18-24 hours.

Post-Hybridization Washing

[0157] To obtain single stranded cDNA probes on the array, all non-specifically bound cDNA probe should be removed from the array. Removal of all non-specifically bound cDNA probe was accomplished by washing the array and using the following materials: slide holder, glass washing dish, SSC, SDS, and nanopure water. It is highly preferable that great caution be used with the standard wash conditions as deviations can greatly affect data.

[0158] Six glass buffer chambers and glass slide holders were set up with 2X SSC buffer heated to 30-34°C and used to fill up glass dish to 3/4th of volume or enough to submerge the microarrays. It is important to exercise caution in heating of the 2X SSC buffer since a temperature of greater than 35°C might strip off the probes. The slides were removed from chamber and placed in glass slide holders. It is preferable that the slides are not allowed dry out. The slides were placed in 2X SSC buffer but it is recommended that no more than 4 slides be placed per dish. Coverslips should fall off within 2 to 4 minutes. In the event that the coverslips do not fall off within 2 to 4 minutes, very gentle agitation may be administered. The stainless steel slide carriers were placed in the second dish and filled with 2X SSC, 0.1% SDS. Then the slides were removed from glass slide holders and placed in the stainless steel holders submerged in 2X SSC, 0.1% SDS and soaked for 5 minutes. The slides were transferred in the stainless steel slide carrier into the next glass dish containing 0.1X SSC and 0.1% SDS for 5 minutes. Then the slides are transferred in the stainless steel carrier to the next glass dish containing only 0.1X SSC for 5 minutes. The slides, still in the slide carrier, was transferred into nanopure water (18 megaohms) for 1 minute.

[0159] To dry the slides, the stainless steel slide carriers were placed on micro-carrier plates with a folded paper towel underneath. The top of the slides were gently dabbed with a tissue. Then the slides were spun in a centrifuge (Beckman GS-6 or equivalent) for 5 minutes at 1000 rpm. It is very important that the slides do not air dry, as this will lead to increased background.

[0160] When the examples are practiced by a skilled artisan as disclosed, an analysis of a toxicological response to an agent, for example, cadmium chloride, can be obtained as shown in Figures 1 and 2.

Example 13 Reverse transcription of mRNA

[0161] This procedure was used prior to the step of acrylamide gel electrophoresis and after the step of RNA isolation. Reverse transcription of mRNA transforms mRNA to cDNA. The following were used in this procedure:

H-T_{11} Primer:	Arbitrary primers marked either G, A, or C. 2μM concentration.
H-AP Primer:	Arbitrary primer, 2μM concentration. Marked numerically.
RH-T11:	Arbitrary primer, marked either G, A, or C. 2μL concentration.
Thermocycler:	MJ Research DNA Engine, PTC-200 Peltier Thermal Cycler. Programmed at 65°C for 5 min→37°C for 60 min→75°C for 5 min→4°C for the RT reaction. Another program is needed at 94°C for 30sec→40°C for 2 min→72°C for 60 sec→for 40 cycles followed by 72°C for 5 min→4°C.
MMLV:	Reverse transcriptase enzyme, 100 units/μL. One unit is the amount of enzyme that incorporates 1nmole of TTP (Thrombotic Thrombocytopenic purpura) into acid-insoluble form in 10 minutes at 37°C using a Poly-A- oligo(dt)12-18 as a substrate.
Total RNA (DNA free):	Obtained from RNA isolation and purification.
DH_20:	18Ω super filter in glass room.
DNTP mix:	Oligonucleotide mix, containing in 250μM

	concentrations, A, T, G, and C.
5X RT buffer:	250 mM Tris-HCL (pH 8.3), 375 mM, 50 mM DTT, 15 mM $MgCl_2$.
10X PCR Buffer:	100 mM Tris-HCL(pH 8.8 at 25°C, 500 mM KCL, 0.8% Nonidet P40, 15 mM $MgCl_2$.
Taq DNA polymerase:	Enzyme used in PCR amplifications, 1000 units 5U/μL.
RT Mix:	Obtained from the reverse transcription(RT) of mRNA.
Pipette:	VWRbrand®; Pipette tips: ART® 10 REACH™, 20P, 100P, 200P, 1000E.
Set-up:	Countertops were wiped down with 70% ethanol prior to experiment. All tubes and pipettes were sterile before start.
General set-up:	The reagents were arranged in ice container and all tubes to be used in the experiment were labeled.

RT Reaction: (for 20μl final volume)	μL	
dH_20	9.4	
5X RT buffer	4.0	
dNTP Mix	1.6	
Total RNA(DNA-free)	2.0	(.1μg/μl freshly diluted)
H-T_{11}M primer(clear tubes)	2.0	
Total	19.0μL	

[0162] The following procedure was followed: Thaw components and set them on ice. Set up 3 RT reactions for each RNA sample in three PCR tubes. Each tube should contain each of the three different H-T_{11} primers(G, A, or C). A master mix with out the RNA template is recommended to reduce pipeting errors. Duplicate reverse transcriptions for each RNA sample can also be done and run side-by-side on a 1.5% agarose gel. Program a thermocycler to: 65°C for 5 min→ 37°C for 60 min→

75°C for 5 min→4°C . After the tubes have been at 37°C for 10 min, pause the thermocycler and add 1μL of MMLV reverse transcriptase to each tube. Primers and samples are light sensitive and it is recommended that they be kept in the dark. Samples can be stored at –20°C until ready to proceed with the PCR reaction.

[0163] *PCR Reaction (From RNAspectra Red Kit ™from GenHunter)*

For a 20μL final volume	μL
dH20	10.2
10X PCR buffer	2.0
dNTP Mix	1.6
H-AP primer(2 μM)	2.0
RH-T_{11} primer (pink tubes)	2.0
RT Mix	2.0
Taq DNA Polymerase	0.2
Total	20.0μL

[0164] It is important that RT mix contains the same H-T_{11}M primer used for PCR. Master Mixes without the RT mix is recommended to avoid pipeting errors. Program a thermocycler for 94°C for 30 sec→40°C for 2 min→72°C for 60 sec→for 40 cycles followed by 72°C for 5 min→4°C indefinitely. Examples of genes which were subjected to this protocol are shown in Tables 5 and 7.

Example 14 Reamplifying cDNA

[0165] This procedure was used prior to the cloning step and after acrylamide gel electrophoresis. The following were used in this procedure:

DH_20:	18Ω super filter in glass room.
10X PCR buffer:	100 mM Tris-HCL(pH 8.8 at 25°C, 500 mM KCL, 0.8% Nonidet P40, 15 mM $MgCl_2$.
DNTP mix:	Oligonucleotide mix, containing in 250μM concentrations, A, T, G, and C.
H-AP Primer:	Arbitrary primer, 2μM concentration. Marked

	numerically.
RH-T11:	Arbitrary primer, marked either G, A, or C. 2μL concentration.
Thermocycler:	MJ Research DNA Engine, PTC-200 Peltier Thermal Cycler. Programmed at 94°C for 30sec→40°C for 2 min→72°C for 1 min→39 cycles→72°C for 5 min→4°C.
cDNA template:	Obtained from the reverse transcription reaction.
Taq DNA polymerase:	Enzyme used in PCR amplifications, 1000 units 5U/μL.
Centrifuge:	Eppendorf centrifuge 5417C.
Pipette:	VWbrand®; Pipette tips: ART® 10 REACH™, 20P, 100P, 200P, 1000E
Glass plates:	Acrylamide long jumbo gel™ system, Hitachi genetic systems.
Hot plate:	VWR scientific products, Dyla dual hot plate/stirrer.
Set-up:	Countertops were wiped down with 70% ethanol prior to experiment. All tubes and pipettes were sterile before start.
General set-up:	The reagents were arranged in ice container and all tubes to be used in the experiment were labeled.

[0166] The following procedure was followed: Take off smaller glass plate, leaving the gel attached to the larger plate. Lay the glass plate with the gel attached to the paper printout of the gel. Align the printout of the gel with the glass plate, using the tape fragments. Cut out the bands of interest with a clean razor blade, or small bore needle. Transfer each gel slice to a 1.5 ml microcentrifuge tube filled with 100 μL of dH_20. Soak the gel slice for 10 min at room temperature. Make sure the gel slice is completely covered by dH_20. Boil the tube for 15 minutes to elute DNA from the gel slice. Spin the tube for 2 min(~14,000 rpm) to pellet the gel. Transfer the supernatant to a new 1.5 ml tube. Add 10 μl of 3M sodium acetate, 5 μL of glycogen (10mg/ml), and 450 μL of 100% EtOH. Let sit for 30 min in a -80°C

freezer. Centrifuge at 14,000 rpm for 10 min at 4°C to pellet the DNA. Remove the supernatant, then rinse the pellet with 200 µL of *ice-cold* 85% EtOH. Spin 1 min at 14,000 rpm and remove the residual EtOH. Dissolve the pellet in 10µl of dH_20. You can use 4 µL for reamplification. Save the rest in a -20°C freezer.

Reamplification PCR Reaction	µL
dH20	23.4
10X PCR buffer	4.0
dNTP Mix	0.2
H-AP primer, 2µM	4.0
H-T_{11}M(clear tubes)	4.0
cDNA Template	4.0
Taq DNA Polymerase	0.4
Total	40.0µL

[0167] The reamplification was done using the same primer combinations as the PCR protocol of Example 13. 10 µL of sample was added to 3 µL of loading dye and run on a 1.5% agarose gel and then stained with ethidium bromide for confirmation. PCR product was then sequenced. The PCR product can also be used for cloning. A commercial source Sequetech (Mountain View, CA; 1-800-697-8685) was used for direct sequencing. If the samples are to be cloned, they can be stored at –80°C for great periods of time, or used directly for ligations.

[0168] Examples of genes which were subjected to this protocol are shown in Tables 5 and 7.

Example 15 Measurement of IL-8 Dose Response

[0169] MDCK cells were dosed with cadmium chloride for 24 hrs at three different dosages (0.1 µM, 1 µM, and 10 µM) and then RNA was isolated from the cells according to protocol detailed in Example 1. cDNA probes were made according to the methods described herein and the Examples above and IL-8 gene

expression was measured. Figure 3 shows the result of the dosages and that the fold induction varies with the dosage.

Example 16 Erythromycin estolate dosing

[0170] Liver and kidney organs were isolated from a dog which was dosed with erythromycin esolate at 100 mg/kg/day for 2 or 10 days. The dog was euthanized 16 hours after the last dosage and the organs were harvested according to standard sterile procedure. RNA was isolated from the kidney and liver as detailed in Example 1. cDNA was made from the RNA and used as probes as detailed in the Examples above. Results are shown in Figure 4 and 5.

Example 17 Amplification of RNA

[0171] Amplification of RNA is accomplished by using the following protocol:

1. Combine in a microfuge tube:

1ug total RNA/8ul DEPC treated water (Ambion #9922)

2ul (1ug) Oligo $d(T)_{22}$ – T7 (Operon, 5'TCT AGT CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG GCG 3') (SEQ ID NO: 385)

Heat to 70°C for 3 minutes.

2. While reaction is cooling to room temperature to synthesize first strand cDNA add the following:

4ul First Strand Synthesis buffer (Gibco, supplied with SSII)

2ul 0.1M DTT (Gibco, supplied with SSII)

2ul 10 mM dNTP's

1 ul Anti-RNase (Ambion catalog #2690)

1ul Template Switching Primer (1ug/ul) (Operon, 5'-AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG-3') (SEQ ID NO: 386)

2ul Superscript II (Gibco catalog #18064-022)

*cDNA synthesis is completed at 42°C for 60 minutes.

3. Place reaction on ice and add the following:

106 ul dH_20

15 ul Reaction 2 buffer (Gibco, supplied with Klenow)

3 ul 10 mM dNTP's

1ul RNase H (Gibco catalog #18021-014)

1ul T4 DNA Polymerase (Gibco catalog #18005-017)

2ul Klenow (Gibco catalog #18012-021)

*2^{ND} Strand Synthesis is completed by using the 2^{ND} SS PCR program (see appendix).

4. End the above reaction with 7.5 ul of 1M NaOH/2 mM EDTA, heat to 65°C for 10 minutes.

5. Add 15 ul 3M NaOAC and extract with 150 ul Phenol: chloroform: isoamyl alcohol 1:1 (see appendix).

6. Precipitate with 1ul linear acrylamide (Ambion #9520) and 300 ul 100% EtOH (Aldrich #E7023) , put in 95% EtOH/dry ice bath and place at-80°C for 20 minutes or O/N.

7. Centrifuge at 14000 rpm for 10 minutes, decant supernatant.

8. Wash with 70% EtOH, centrifuge at 14000 rpm for 10 minutes and decant again.

9. Let pellet completely air dry and resuspend in 500 ul dH_20.

10. Clean and remove unincorporated nucleotides using a Microcon 100 (Amicon #42412) concentrator.

11. Lyophilize sample to 16 ul using a Speedvac at 45°C.

12. To perform the amplification: 16 ul of ds-cDNA sample is combined with the following: 4ul 10X Reaction buffer, 3ul 100mM dATP, 3ul 100mM dCTP, 3ul 100mM dGTP, 3ul 100mM dTTP, 4ul .1M DTT, 4ul T7 enzyme mixture

(Ampliscribe T7 Transcription kit, Epicentre Technologies #AS3107) for a total volume of 40ul.

13. Incubate at 41°C for 4-5 hours.

14. Place sample in a 1.5 ml centrifuge tube and bring volume up to 100ul by adding 60ul DEPC treated water.

15. Extract with 100ul Phenol: chloroform: isoamyl alcohol 5:1.

16. Precipitate with 1ul linear acrylamide (Ambion #9520) and 300ul 100% EtOH (Aldrich #E7023), put in 95% EtOH/dry ice bath and place at-80°C for 20 minutes or O/N.

17. Centrifuge at 14000 rpm for 10 minutes, decant supernatant.

18. Wash with 70% EtOH, centrifuge at 14000 rpm for 10 minutes and decant again.

19. Let pellet air dry and resuspend in 100ul DEPC dH_20. To verify products: OD sample and run on a formaldehyde gel.

20. Store samples at -80°C. About 300-400ug aRNA can be expected from 1ug total RNA

Example 18 Anti-sense RNA probes

[0172] 1. Combine in a microfuge tube:

20ug aRNA(1x) /8ul DEPC water (Ambion #9922)

6 ul Random Hexamers (Gibco #48190-011)

Heat to 70°C for 10 minutes, put on ice for 2 minutes.

[0173] 2. Keep reaction on ice while adding the following:

20ul First Strand Buffer (Gibco, supplied with SSII)

10ul 0.1M DTT (Gibco, Supplied with SSII)

10ul 10mM dNTP's

4ul Superscript II (Gibco #18064-022)

2ul Anti-Rnase (Ambion #2690)

Heat to 41°C for 60 minutes

[0174] 3. Add 8ul Oligo $d(T)_{12}$ (1ug/ul) to the reaction.

[0175] 4. Heat to 70°C for 10 minutes, put on ice for 2 minutes

[0176] 5. Place reaction on ice and add the following:

38ul DEPC water

30ul 2^{ND} Strand Synthesis buffer (Gibco, supplied with Klenow)

6ul 10mM dNTP's

2ul Rnase H (Gibco #18021-014)

2ul T4 DNA Polymerase (Gibco #18005-017)

4ul Klenow (Gibco #18012-021)

2ul 0.125mM CyDye (Cy3 or Cy5, Pharmacia #27-2692-01) for a total volume of 150ul.

[0177] 6. Place in PCR machine and use program 2^{ND}SS (see Appendix).

[0178] 7. Place reaction into a 1.5ml centrifuge tube and add 600ul of precipitation mix (see appendix). Place in -80°C for 1 hour.

[0179] 8. Centrifuge for 10 minutes at 14,000rpm

[0180] 9. Decant supernatant

[0181] 10. Wash with 500ul 70% EtOH. Centrifuge again.

[0182] 11. Decant supernatant, and let samples air dry.

[0183] 12. At this point you can resuspend samples in dH_20 to read fluorescence or go directly to hybridization.

APPENDIX

[0184] Phenol Chloroform Extraction is performed as follows:

1. Add 1 volume of Phenol: chloroform: isoamyl alcohol (use 5:1, Sigma # P-1944, if extracting RNA and 1:1, Sigma #P-2069, if extracting DNA).
2. Vortex to mix.
3. Centrifuge at 14000 rpm for 5 minutes.

4. Remove the upper, aqueous layer to a clean 1.5ml centrifuge tube, be careful to avoid the denatured proteins which are found at the aqueous/phenol interface.

5. Centrifuge tube again and remove any more aqueous layer that may be present.

6. Follow with precipitation.

2^{ND} SS PCR Program is as follows: 37°C for 2 minutes → 95°C for 3 minutes → 65°C 3 minutes → 75°C 30 minutes.

Precipitation Mix is as follows: 68ul water, 92ul ammonium acetate (filtered), and 440ul 95% EtOH.

To use the Microcon 100 concentrator, the following is done:

1. Prepare column with 500ul dH20 centrifuge at 3000K for 10 minutes
2. Add sample to column and centrifuge at 3000K for 10 minutes
3. Invert column into 1.5ml collection tube and centrifuge at 4500K for 3 minutes.